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# Stem Cell Transplantation

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## PREFACE

*"All that lives must die, passing through nature to eternity"*  
—Hamlet (*W. Shakespeare*)

Organ transplantation has been the most important therapeutic advance in the last third of the 20th century. Its development has revolutionized medicine, as demonstrated by the fact that a large number of researchers in this field have been awarded Nobel Prizes.

In the beginning of this century, we are witnessing with great expectations the emergence of a new field of medicine related to the arrival of a new player on the scene: "**stem cells**" and their potential use in regenerative medicine. This volume aims to cover important aspects of the various facets of organ transplantation and regenerative medicine, with leading specialists in these fields setting out their vision. We try to rigorously explain current and novel scientific research in these fields—areas which arouse great interest from society in general, due to their potential use in modern medicine for the treatment of a great number of diseases.

The chapters of this volume are divided into four sections, **Section I** being devoted to the basic aspects of transplantation (immunological and pharmacological). A century has passed since the field of immunology was officially recognized with the award of the 1908 Nobel Prize to Elie Metchnikoff and Paul Ehrlich. They each developed concepts and points of view which led to the establishment of this new scientific discipline. Metchnikoff described the mechanism of phagocytosis and is considered the father of the field of cellular innate immunity. Ehrlich described the basis for acquired immunity through the production of antibodies. These two findings have become the cornerstones of this discipline, which has advanced very rapidly since the last century, providing an in depth understanding of the mechanisms related to organ transplant rejection. The first functioning transplants were of kidneys from living donors in the 1950s. During the '70s and '80s, the practice of organ transplantation stopped being regarded as an experimental therapy and became a reality in hospital medicine. Sir Peter Medawar pointed out that the rejection of transplant organs by the recipient body was mediated by an immunological reaction which could be modified. This was due mainly to the identification of the major histocompatibility complex (MHC) and the characterization

of the so-called human leukocyte antigen (HLA). On the other hand, the discovery of effective immunosuppressants (such as cyclosporin) meant a dramatic improvement in survival for patients and for grafts.

There are two main differences between organ transplantation and any other type of surgery. One concerns the need for organs to be donated by a living person or received from a deceased donor, and the other is the requirement for lifelong immunosuppressive medication in transplanted patients, with the associated risk of becoming vulnerable to infection and development of chronic diseases. Rejection is, as such, inherent to transplantation, and the fight against it is an important part of the history of this therapy. The medium and long-term challenges consist of overcoming so-called chronic rejection and finding a way to prevent it. The understanding of the mechanisms of immunogenicity and immunological tolerance is allowing the development of a new generation of immunosuppressive agents which manipulate the immune system more precisely. Clinical trials are being undertaken to refine existing regimens.

The development of immunotolerance is, in a strict sense, the acceptance of the graft by the recipient without immunosuppressive medication. Several multicenter trials including Clinical Trials in Organ Transplantation (CTOT), a cooperative research program sponsored by the National Institute of Allergy and Infection Diseases, and Reprogramming the Immune System for the Establishment of Tolerance (RISET), an ongoing collaborative effort across the European Union are aimed at minimizing immunosuppression. Other future possibilities are open with the potential use of immunotherapeutic techniques that inhibit recognition by the immune system of the transplanted organ through the development of combined cell therapies (for example, generation of regulatory T-lymphocytes and mesenchymal stem cells).

However, despite this progress, the mismatch between supply and demand is, by far, the biggest problem in the world of transplants. Spain, the world leader in organ donation, has managed to avoid an excessive growth in waiting lists, but is still not capable of meeting all the demand for organs. One strategy proposed has been the use of animal organs. However, in order for xenotransplants to work, researchers must find the way to block immunological rejection. Moreover, at present, there is a moratorium on their use, based on the risk represented by the infection of humans with animal viruses.

During the last few years, we have seen the growth of a new area of knowledge: regenerative medicine. The scientific developments deriving from the discovery of so-called “stem cells” have raised great expectations given their potential use in tissue replacement. Tissue or cell transplants have become a potential therapeutic replacement for organ transplantation in the future. A “stem cell” is one that is undifferentiated and able to produce the specialized cells of various tissues and organs. Furthermore, these cells are capable of proliferating and growing in cultures indefinitely, which has been described as “cell immortality”. Many cell types fall within this definition of stem cell.

This volume deals mainly with the so-called embryonic and adult stem cells. The latter are the subject of **Section II**. The first effective cell therapy treatments were carried out using haematopoietic stem cells (HSCs) for bone marrow transplantation. HSCs are unique in their ability to migrate to various sites, ensuring the safety and integrity of their regenerative potential. Different chapters focus on the guidance cues and molecular pathways regulating HSC trafficking throughout the lifetime of the organism. Moreover, we know now that there are other places in the body where there are reservoirs of

adult non-haematopoietic stem cells. The presence of a small subpopulation of adult stem/progenitor cells in most tissues and organs provides the possibility of stimulating their *in vivo* differentiation potential, or of using their *ex vivo* expanded progenies for cell-replacement and gene therapies with multiple applications in humans without high-risk of graft rejection and major side effects.

An adult stem cell, therefore, has the possibility of self-renewal and of generating certain types of cells (multipotent stem cells) indefinitely, depending on their location, such as: cartilage, adipose, connective and muscle tissue. In relation to this, molecular signals of specific niches or microenvironments play a fundamental role in the maintenance of the differentiation potential. At present there is a drive to search for the factors that trigger the processes of regeneration from the stem cells in various locations in the body and how to control them. One of the emerging fields of research today is based on the existence of “tumour stem cells” and their implications in cancer development. Their similarity with adult stem cells will provide us with a new approach for the treatment of certain types of cancer.

**Section III** of this volume focuses on the description of embryonic stem cells. Pioneering work carried out by James Thomson (1998) demonstrated the possibility to derive embryonic stem cells from a human blastocyst for the first time. This was a milestone, opening a new field full of potential. The possibility of inducing these cells to differentiate into any type of tissue (pluripotency) and of having unlimited quantities of them provide new hope for treatment of many diseases. Also, the development of cloning techniques enables cells genetically identical to those of the patient to be derived, thus avoiding the risk of immunological rejection, a strategy commonly termed “therapeutic cloning”. Several research groups have published studies on “cell reprogramming” techniques. For this purpose, reprogramming of somatic cells into pluripotent stem cells (iPSC) has been achieved by introducing four transcription factors in 2007 by Shinya Yamanaka’s team. These results opened the possibility of using adult somatic cells and transforming them into stem cells similar to embryonic stem cells. Induced pluripotent stem cells overcome the problem of immune tolerance and the ethical issues faced by the use of allogenic embryonic and adult stem cells in patients. Applications of patient-specific induced pluripotent stem cells have focused on disease modeling, drug screening and therapy. Recently, iPS cells have been derived from patients with a variety of genetic diseases, such as Parkinson’s, Huntington’s, Down syndrome, muscular dystrophy, amyotrophic lateral sclerosis and others.

**Section IV** summarizes some ongoing approaches of stem cell regenerative medicine and also introduces recent findings from published studies and clinical trials. It is necessary to evaluate and demonstrate its effectiveness, biosecurity and real capacity to repair tissues that have been damaged by various conditions, such as neurodegenerative, cardiovascular and endocrine diseases, before their use in therapy. Some cell therapies had been established and approved for clinic use, such artificial skin, keratinocytes, chondrocytes, liver, cells of the corneal limbus and others. The present status of tissue bioengineered techniques in the regeneration of different organ systems is also reviewed. Synthetic biomaterials used to create scaffolds for tissue engineering applications have been limited in large part due to the lack of specific cell binding motifs that would allow cells to function properly. Creation of custom-made bioengineered organs by using scaffolds as decellularized native tissues seeded with autologous cells remains a major challenge.

Indeed, there are still many basic aspects to investigate and develop, such as: the specificity of molecular signals involved in the differentiation of stem cells into various cell lineages, the use of specific biomaterials supporting organogenesis and the development of biosecure gene therapies which can be transferred to clinical settings.

It is hoped that in the coming years better insight into the mechanisms underlying the beneficial effects of progenitor cells will help the design of more specific targeted therapies.

We would like to acknowledge the authors for their excellent contributions.

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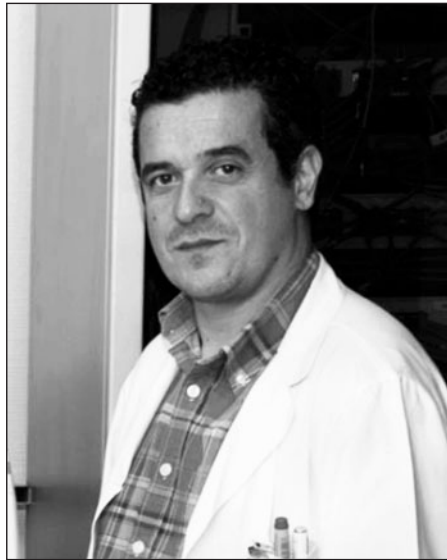


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## CHAPTER 1

# THE FRONTIERS OF ORGAN TRANSPLANTATION AND CELL THERAPY

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**Abstract:** The biggest problem in the area of organ transplantation is often the mismatch between supply and demand. Extrapolating the transplant waiting lists in Spain at the end of a year to the global population, more than one million people would be able to benefit from a transplant if there were enough available organs and adequate infrastructure. The first frontier and the most important is therefore the donation of organs. The aim of this chapter is to set out the most notable points concerning the various themes (donation, rejection, xenotransplants, tissue transplantation and stem cells therapy), and describe new avenues to be explored: The frontiers that it will be necessary to cross in order to continue the progress in saving lives and improving the health of hundreds of thousands of people across world.

In the last years, embryonic stem cells have become in the great hope of many millions of patients across the world. In theory, the possibility to have unlimited quantities of these cells, to culture them, and to make them differentiate into cells of the liver, nervous system or heart would in fact become the ideal solution for the treatment of millions of patients. It is quite plausible that what organ transplantation has represented in the 20th century, bringing down taboos and saving hundreds of thousands of lives, is going to be replaced by stem cell therapy in the 21st Century.

## INTRODUCTION

In a book-like this, in which there are numerous references to the so-called “classical” transplantation protocols, as well as the new therapeutic approaches that are to become an alternative for a multitude of processes in the near or distant future, it seems reasonable to devote this first chapter to present an overall vision of the subject. The aim is to set out the most notable points concerning the various themes (Table 1), and above all, describe

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**Table 1.** Frontiers to define the donation and transplantation of organs, tissues and cells

- 
1. Availability of organs for transplantation:
    - a. Donation after brain death.
    - b. Donation in cardiorespiratory arrest.
    - c. Living donor transplant.
  2. Prevention and management of rejection.
  3. Xenotransplants.
  4. Organ transplant.
  5. Tissue transplant/tissue engineering.
  6. Cellular transplant/cellular therapy.
- 

new avenues to be explored: The frontiers that it will be necessary to cross in order to continue the progress in saving lives and improving the health of hundreds of thousands of people across world.

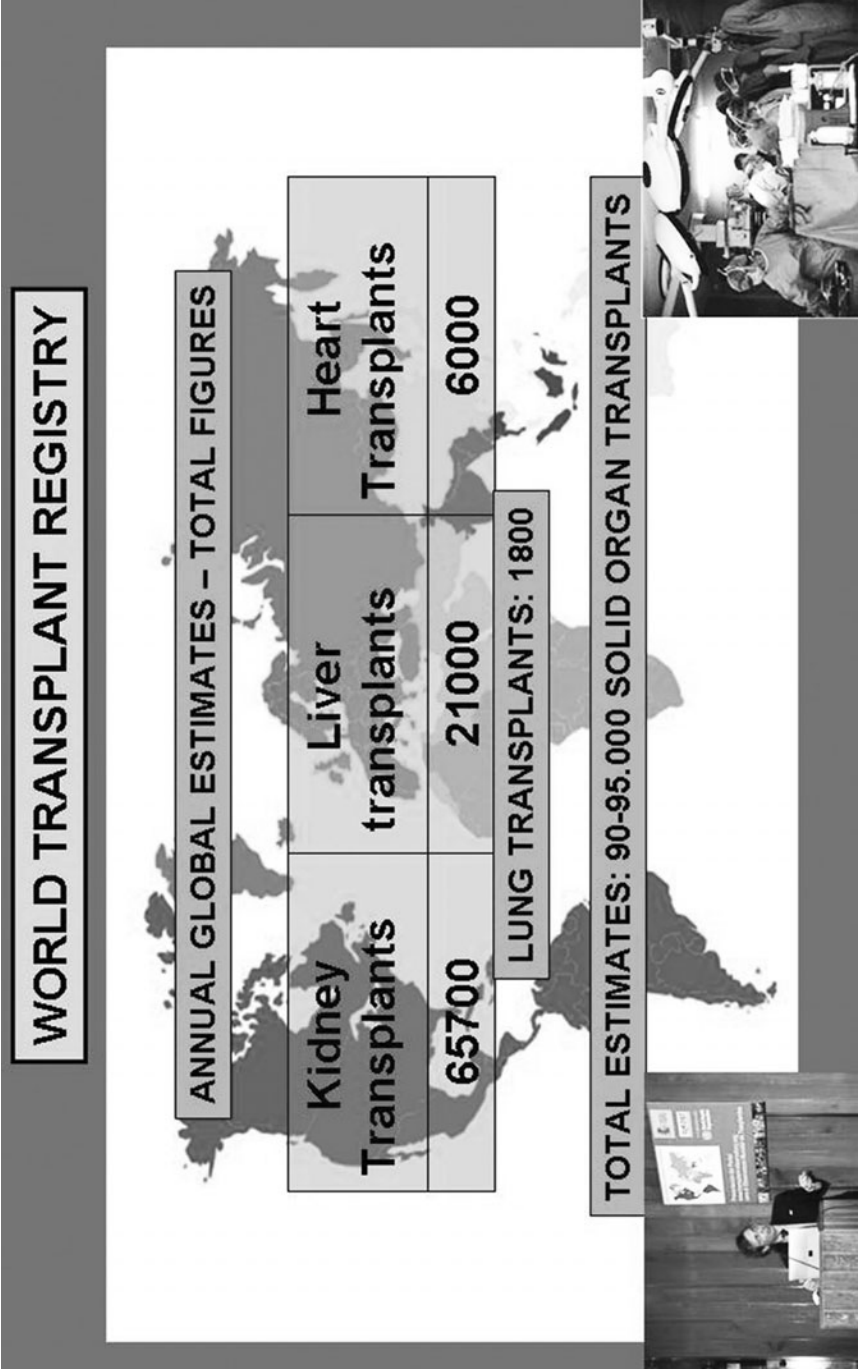
## AVAILABILITY OF ORGANS FOR TRANSPLANTATION

The biggest problem in the area of organ transplantation is often the mismatch between supply and demand. In contrast to this limiting factor, the other problems, that we will go on to present as frontiers to cross, remain for the moment mere details. According to the data collected jointly by the Spanish National Transplant Organisation (*ONT*) and the World Health Organisation for the Global Observatory of Donation and Transplantation,<sup>1</sup> every year around 95.000 patients are recipients of solid organ transplants (excluding tissue and cell transplantation) (Fig. 1). It is not possible to know the exact demand for transplants due to the lack of an official waiting list and indeed of any transplant organisation covering a good part of the world. However, if we extrapolate the transplant waiting lists in Spain<sup>2</sup> at the end of a year to the global population, there would be more than one million people able to benefit from a transplant if there were enough available organs and adequate infrastructure. In other words, overall less than 10% of the candidates for this treatment obtain the organ needed and transplant policies are inevitably conditioned by the enormous mismatch that this represents.

The first successful transplants were those of kidneys coming from a living donor,<sup>3</sup> dating date back to the nineteen-fifties. However, it was the deceased donation which made possible the major development of all types of transplants, especially that of vital organs. The problem is that deceased donation has a fundamental limiting factor: The epidemiology of brain death, itself determined by a series of structural issues (availability of beds in intensive care units, number of respirators, general healthcare resources) and organisational factors.

The first frontier to target, and perhaps the most important one, is therefore the donation of organs. The remaining hurdles are of lesser importance since if there are no organs for transplantation, there is no point in anything else. Transplantation is the only medical speciality that in treating patients is dependent on another individual, dead or alive, who makes it possible by giving their organs. As regards donation of deceased-donor organs, since the establishment of the *ONT*<sup>5-9</sup> in 1989, Spain has been gradually pushing back the frontiers. Specifically, Spain has been progressively improving its organ donation





**Figure 1.** World Transplant Registry WHO/ONT: Overall figures for organ transplant worldwide. Reproduced with the permission of the Global Observatory on Donation & Transplantation (<http://www.transplant-observatory.org>).

system and has reached and maintained 33-35 donors per million people (pmp), a level never achieved by any other country in the world. Since 1992, we have led the ranking in both generosity and organisation, but this should not lead to self-complacency and we should continue to strive for better results. In fact, although a national record of 1.606 donors was achieved in 2009, with a 34.4 donors pmp, 3-5 out of the 17 Spanish Autonomous Regions usually reach levels above 40 donors pmp (2,9). Specifically, a small region as is La Rioja could boast of 74.2 donors pmp in 2007, and others such as The Basque Country, The Canary Islands and Cantabria have exceeded 50 on several occasions. From this, we can state that with optimum organisational and structural conditions such as achieved in these Autonomous Regions, it should be possible to obtain similar figures elsewhere. Indeed, some Italian regions such as Toscana, that have adopted a model similar to ours, have also reached 40 donors pmp.<sup>10</sup>

Therefore, the ONT<sup>2</sup> has set as an objective achieving 40 donors pmp for the whole of Spain, an initiative called “Plan donación cuarenta”. This would be a new achievement for Spain but also set the “Gold Standard” to be reached internationally, in which all the strategies with proven efficacy are put together to improve organ donation rates.

Up to now we have been talking about the typical brain-dead donors, who make all types of transplants possible, and who are obviously closely linked to intensive care units. These represent no more than 2-3% of those who die in a hospital, hence the interminable mismatch between supply and demand in this therapeutic procedure.

However, there is another minority group: Nonheart-beating donations or more descriptively donation after cardiac death (DCD). It involves those patients who suffer from cardiac arrest of any cause, and despite the usual procedures cannot be successfully resuscitated, leading to a shortage of oxygen in the central nervous system and the resulting irreversible damage. After all, from the beginning of time, this has always been and still is the most common way to die. Donation and subsequent organ transplant can only be achieved if at this moment, procedures for the cannulation and perfusion of organs are carried out.

This type of DCD, also referred as “uncontrolled” because it is not possible to know when they are going to happen, require an agile and efficient organisation. They represent just over 5% of Spanish donors, although in Barcelona and especially in Madrid, this percentage is much higher.<sup>2,11</sup> On the other hand, a new type of donor started to appear a few years ago for the world transplant community. These are the so-called controlled DCD or Maastricht Category III donors: These are people with irreversible brain injury, who require mechanical ventilation but who are not brain dead. In this situation, and after informing relatives and receiving their consent, life-support is withdrawn until the heart stops, the individual dies and organs can be donated.<sup>12,13</sup> This form of donation, excluded in Spain in the Consensus Conference held in 1995 which opened the way to other DCD, although legally possible, is not being performed in Spain and most countries in Southern Europe and Latin America. On the other hand, it accounts for a third of the donations in The Netherlands and about 10% in the United States, where it represents the fastest growing-type of transplant, and there has been a similar pattern in other countries such as United Kingdom and Australia. Whether through the increasing uncontrolled DCD in large hospitals connected to good emergency services, or through the generalisation of controlled DCD, which despite all the ethical and legal issues for many countries and cultures, currently represents one of the greatest avenues of expansion worldwide in organ donations from deceased donors, the truth is that cardiac arrest deaths represent a

non insignificant way of increasing the number of donors, and therefore in the number of people who can benefit from these therapies.

A similar statement can be made with respect to live donation. In the nineteen-eighties there was a great development of donation from deceased donors, to the point that it was thought that live donations were going to become almost insignificant. Indeed, the Council of Europe recommended reducing its use as far as possible in a resolution of the Council of Ministers in 1987, as did the World Health Organisation in the early nineties.

However, things have not evolved as some expected. With the exception of Spain, where the donation of organs arising from deceased donors has not stop increasing since the late eighties, there is a growing mismatch between offer and demand for all types of transplant, but especially for renal transplants. That is why, given the improvement in the outcomes as a consequence of better drugs against rejection and the increase in medical recommendation for transplants that cannot be carried out in other ways,<sup>14</sup> the opinion of the transplant community and of the society in general has changed. These days, live transplantation has stopped being seen conflicting with deceased donor transplantation and has become a growing complementary alternative in countries. There is little doubt that for a given country, it is much easy to have a live kidney transplant programme than having a system for deceased donor kidney allocation that works. It requires only a relatively simple technique which does not need major facilities.

The consequence is that in several countries in Asia, Africa and, to a lesser extent, Latin America almost 100% of the renal transplants come from living donors. They represent 42% of the total in Latin America, 44% in Australia, and 36% in the United States, where there has been a real growth in this type of treatment. In the European Union they account for just 17%, with higher percentages, over 30-35%, found in northern countries and in Greece, and lower rates in Latin countries, accounting for 10% in Spain, although there has been an increase in recent years as a consequence of an active promotion policy.

While we are not going to analyse them here, the potential for expanding live donation, applicable not only to kidneys but also to the liver and, though to a much lesser extent, to other organs such as the lung, pancreas and intestine, are theoretically unlimited. However, in practical terms, there are two basic limiting factors: The potential damage to the donor and the commercialisation of organ donation, which according to the WHO affects 10% of all the live transplants carried out worldwide.

One hundred experts from 40 countries met in April 2004 in Amsterdam. The objective was to establish an international consensus on the standards that should be guaranteed to kidney donors.<sup>15</sup> The necessity to minimise the physical, psychological and social risks for the donor from the removal of a kidney, at the same time as optimising the benefit to the recipient and maintaining the public confidence in the donation process. The core of the document approved contains the recommendation of a thorough evaluation of potential donor's health to avoid any subsequent complications, as well as the need to provide extensive and detailed information on all the risks of the intervention in the short, medium and long-term, and the potential alternative treatments for the potential recipient. Another of the recommendations was the careful monitoring of the donor in order to detect any emerging complications, in to maximise the chances of successfully addressing the problem, as well as improving our understanding of the possible impact of the removal of a kidney. This is a very reasonable document, and its recommendations, should they be implemented in full, would go a long way towards eliminating the abuses currently occurring in many parts of the world. More recently, experts and organisations all over

the world have agreed the so-called “Declaration of Istanbul”<sup>16</sup> which represents a clear consensus against the commercialisation of transplantation and transplant tourism.

The combination of the three forms of donation: Donation after brain death, donation after cardiac death and live donation currently enable 100.000 organ transplants every year.<sup>1</sup> There is no doubt that one of the frontiers to cross is improvement in these three modalities, to make these types of treatment available to anyone who needs them.

## **PREVENTION AND MANAGEMENT OF REJECTION— IMMUNOTOLERANCE**

Rejection is inherent to the transplantation procedure, and the fight against it has been a key issue in the history of transplant therapeutics. Initial rudimentary immunosuppressive protocols have been replaced by current protocols in which drugs, discovered in recent decades, are increasingly combined in a personalised manner. It can be asserted that avoidance of short-term rejection is almost guaranteed with the use of current medications. This explains the excellent functional survival rates of the various grafts and indeed of the recipients for the majority organ transplants. Graft failure in the early months, although it may have an immunological basis, is mainly due to problems with respect to organ viability, the consequence of a greater use of donors with risk factors, as well as to surgical and infectious factors, and others associated with the clinical status of the recipient.

The challenge, the real frontier of immunosuppression, lies in the middle and long-term in two respects: To extend the good initial results to longer periods of time and to minimise the acute and chronic toxicity that goes with immunosuppressive treatment. The real problem of all types of organ transplant is the progressive functional deterioration, a consequence of so-called “chronic rejection”. This is a complex and poorly understood phenomenon that causes the loss of a great number of grafts in the long-term and is associated with a notable morbimortality, as well as requirement for retransplantation. Another issue is the inherent toxicity associated with the long-term administration of the drugs, leading to a higher rate infections, tumours, metabolic disorders, etc. Evidently, all these problems are largely outweighed by the great clinical and social benefit of transplantation, but they do represent a serious problem that restricts long-term survival and quality of life of many patients. In fact, these have become the real sticking point in the clinical management of the transplanted patient.

The problems mentioned above could be largely solved if we understood the phenomenon of chimerism better.<sup>17</sup> This is a situation in which a transplanted organ or tissue, coming from the same or a different species, instead of being the object of a rejection immune response against the transplant, is accepted as if it has been always part of the recipient. This event occurs spontaneously very rarely and its mechanism still remains largely unknown.

This type of “immunotolerance” has attracted the attention of many experts due to the future importance that its better understanding and control may have for the prognosis of transplanted patients. Another factor is the huge costs of these drugs. It is estimated that in the European Union, the annual expenditure is around 2.000 million Euro, with an average cost per patient between 6-9.000 Euro.

For a long time it has been known that in most cases transplanted patients who abandoned their medication whatever stage in the process lose the organ. Nevertheless, there is a proportion that progress well with no medication, thanks to the mechanism

of immunotolerance mentioned above. If the treatment is dropped on a patient's own initiative, little can be said, but removing the medication of a transplant recipient entails a risk of rejection, a responsibility that cannot be assumed by others. The development of a laboratory test that identifies these patients would be of great value in order to avoid running this risk, and this is subject of research in many groups both in Europe and in the United States.

However, research in this field is not only centred on the identification of potential immunotolerant patients, but also on inducing such a status.<sup>17,18</sup> The most promising lines of research indicate that the transplantation of haematopoietic precursor cells of the donor themselves may be a way of inducing the desired immunotolerant status. This has been tried several times, for example, in the face transplantation carried out in France. However, currently, the unknowns by far exceed knowns. To date, anti-rejection drugs have been aimed at "blinding" the immune system. In this case, we would be intending to retrain it so that learns to co-exist "peacefully" with the new organ: A chimera in all the senses of the word.

## XENOTRANSPLANTS

The concept of transplanting organs of other species' into human recipients is not new. In fact, we could state that there was an instinctive attempt in doctors to try to save the lives of people suffering from renal, liver, and cardiac insufficiency long before the early foundations of modern surgical procedures were laid.<sup>20</sup> In the nineteen-nineties, there was the belief that xenotransplantation into humans might become the solution for the deficit of organs of deceased donors pig being the species with highest potential to become the ideal source of organs for humans.

Indeed, the production of transgenic pigs, that expressed in their cells human complement regulatory proteins, allowed the immunological barrier of hyperacute rejection to be overcome. Until then, this had been the prime limitation of pig organ transplantation into humans. However, in order to avoid these xenografts being rejected later, the use of an intense immunosuppressive treatment is necessary, and this is associated with severe toxic effects in the recipient. The next step is to determine if rejection could be avoided, with acceptable secondary effects for the recipient, using conventional treatments. If not, it might be necessary to resort to new genetic modification in the animals to be the source of the organs, or else to some other type of manipulation in the recipients. Only after we have solved these issues could long-term survival rates (more than 6 months) of pig organs in nonhuman primates be possible. It would then be possible to study the functioning of organs as well as the transmission of infectious agents.

At the end of last century, with the research at that stage of development, the process halted after the publication of results demonstrating infection of human cells by pig endogenous retrovirus and subsequent capacity for recombination. Although there have been no new indications of the danger of this retrovirus, there is a consensus that we should be cautious when considering clinical trials involving xenotransplants. This issue stopped quite a few lines of research; some others remained active, especially those based on cells rather than entire organs (pancreatic islets, hepatocytes, etc.).<sup>20</sup> There has been a lot of progress in the preparation of protocols for the prevention of diseases both in animal carers and potential recipients (especially the latter), and evidence suggests that, after a long pause, we may observe a new dawn in research on this fascinating subject.

To summarize, in the nineties xenotransplantation was seen as the great hope to overcome the lack of human organs available for transplantation. Unfortunately, it seems that it is going to be just that, a hope, for the foreseeable, since the great expectations of the scientific community have not yet been realised. We can just cross one's fingers that the substantial financial and research efforts made to date, will not end in disappointment if we reach a dead end.

## ORGAN TRANSPLANTATION

Generally speaking, the limitation of the various types of transplants is that of achieving extended long-term survival rates. As said previously, the prevention and treatment of rejection in short-term has been fairly satisfactorily addressed, but not for the medium and long-term. This also entails a significant iatrogenic effect, that together with the possible re-appearance of the disease that lead to the final sclerosis of the organ and other factors, limits the long-term functional survival of the grafts and the patient. In Tables 2 and 3 list the maximum survival periods recorded in transplant patients in Spain and across the world for the various organs. The figures show how much has been achieved since the nineteen-fifties regarding these therapies. Further, it is more than likely that the operations being carried out in the present day will achieve even higher survival rates. The boundaries are getting closer all the time.

For each organ, there are certain challenges specialists are working on, in order to optimize the offer of organs and improve outcomes, both from the medical and surgical points of view. Apart from the immunosuppression guidelines that combine several drugs as mentioned earlier, it is worth mentioning the attempts to make better use of organs that otherwise would be dubiously useful: The double kidney transplant in cases of kidneys with reduced renal mass due to age or other factors, "domino" transplants in the case of liver and heart-lungs, the "split" transplants in the case of liver, and other possibilities that arise every day. Even in fully consolidated techniques, transplantation is a combination of the soundness of the results and ongoing innovation to address new challenges. Standing still is not compatible with organ transplantation.

Of all the organ transplants, the only one that still today has not been consolidated is that of the intestine. Despite the fact the first of these being carried out in the USA in the nineteen-sixties, there have been no satisfactory outcomes until recently. In Spain, the first operation was performed in October of 1999 and at the time of writing this paper, he recipient child is perfectly healthy. Only 10-15 operations are undertaken every year in our country, children and adults combined. These are carried out in isolation or in combination with liver or other abdominal organs. At this point it may be worth making

**Table 2.** The longest survival of patients transplanted in Spain

- 
1. Kidney transplantation: 37 years.
  2. Liver transplantation: 21 years.
  3. Heart transplantation: 22 years.
  4. Gut transplantation: 7 years.
  5. Panchreas transplatation: 18 years.
  6. Lung transplantation: 14 years.
-



**Table 3.** World longest survival of transplanted patients

- 
1. Kidney transplantation: 43 years.
  2. Liver transplantation: 36 years.
  3. Heart transplantation: 27 years.
  4. Gut transplantation: 16 years.
  5. Pancreas transplatation: 24 years.
  6. Lung transplantation: 15 years.
- 

some reference to the so called “clusters” or multiple transplants, that may account for 6 or 7 organs from the same donor to the same recipient. They represent final frontiers of organ transplantation, and are subject of frenzied attention from the press due to their spectacular nature. The same patient could receive a liver-stomach- small intestine-large intestine-pancreas and kidney. It should be noted that the situations when such interventions are required are fortunately very rare and the prognosis is poor, in part due to the difficulty of both this type of surgery and complications in the postoperative period.

## TISSUE TRANSPLANTATION/TISSUE ENGINEERING

A fundamental characteristic of tissues is the reason that the process of obtaining them is not under such time pressure as that organs. It is not necessary that the heart is still beating, unlike organs, and this facilitates the procedure a great deal. What is more, they can be preserved for some time, normally including freezing, which makes things even easier. That is why the pending issues for tissue transplantation are not specifically related to the lack thereof, the shortage of them being less pressing than that of organs, in relative terms. Rather, the new challenges involve the need to develop a more effective surgical procedure, with the highest levels of quality and safety and in the precise quantities and sizes required by each patient.

In this context, tissue engineering<sup>21</sup> with an almost infinite possibility of combinations of biomaterials with tissue and stems cells, is nowadays one of the most promising fields of expansion in modern medicine. It is already possible, with more or less difficulty, to reproduce tissue types including skin, osteo—articular tissue, vascular segments and corneas, and now even some organs such as the bladder.<sup>22</sup> These techniques are still in the experimental phase but have enormous potential as regards being able to avoid the lack of raw material to be transplanted and the rejection phenomenon: In many cases it is possible to obtain material from the patient’s own cells and therefore suitable for him/her.

Within tissue transplantation, it is necessary to mention composite tissue allografts: That of forearm and hands and of face transplantation.<sup>23,24</sup> They have in common that many tissue types are transplanted at the same time and therefore are subject to their own problems. Since the first impressive media coverage in 1998, around 30 unilateral or bilateral upper extremity transplants have been performed, three of the latter in Spain, and ten face transplants in France (five), Spain (two), USA (two) and China (one). Despite the beginnings having been quite tortuous, due to factors above and beyond the medical-surgical process, outcomes are generally good, and it seems that they are going to become much more common in the future. While this type of procedure still in an experimental phase and the nerve regeneration is clearly incomplete, the truth is

that transplanted patients are able to move the hands and fingers “teaching” the muscles of the natural arm to pull the newly connected tendons. Even the detractors must admit that during interviews with patients and their families, they have been impressed by the psychological benefits from the transplants, especially for those who had lost both hands.

In any case, the scientific knowledge acquired through these procedures is already impressive. An examination of the motor function before and after the operation of the first double transplant, carried out in France, showed a re-organisation of the zone in such way that areas responsible for other parts of the body spread to occupy what the missing arms had left disused. However, four months after the transplant, the situation had reversed to the situation prior to amputation. These physiological findings are really interesting, and would have only been made thanks to the implementation of this type of treatment. This is yet more evidence that transplants, not only save or improve the lives of many patients, but also contribute to the advancement of scientific knowledge and progress in many fields of medicine.

Face transplantation, in an earlier stage of development, is just as much a challenge, from the surgical, immunological, psychological and even bioethical point of view. The procedure, a display of microsurgery, consists of first removing all the face skin of the recipient patient and exchanging it for a layer obtained from the deceased donor, that includes, not only the skin, but also fat, part of the muscles, blood vessels and nerves. Naturally, everything has to be connected and adapted to the new bone structure of the patient, who clearly keeps their original bones and muscles, while all the covering from the neck to the forehead would be from the donor. The result is then a mixture between the face of the donor and that of the recipient, depending on the quantity of muscle transplanted, although as stated by the supporters of the technique, the facial features depend above all on the bone structure which remains unaltered in this procedure. The fact that the main desired effect is aesthetic, means that any rejection, infection or other complication with such a sensitive area, which could permanently disfigure the graft, might make the cure worse than the disease leading to unforeseeable psychological problems in the recipient. In any case, this represents a new scientific frontier opening up thanks to transplantation.<sup>24</sup>

## **CELL TRANSPLANTATION: CELL/STEM CELL THERAPY**

The first effective treatments using cell therapy in the broad sense were carried out using haematopoietic stem cells from bone marrow. Progenitor blood cells represent at present 98% of human cell implants, despite the notoriety of the other 2% thanks to media coverage. It has been used as a therapeutic procedure worldwide since 1958 when in Paris the first five cases were treated using this technique to address haematological, immunological and tumour diseases.<sup>25</sup> The outcomes depend above all on the disease with which they are used, but there are already survival periods of more than 20 years for diseases that otherwise would be fatal. The research front now opened up, there is enormous potential for improvement in this field. As an example, there is the real possibility of curing some congenital enzyme deficiencies by selective transplant of the normal cells, which was unimaginable a short time ago and which can involve the deliberate conception of a sibling without this deficiency as a source of cells for transplantation. Indeed, whole range of possibilities for many diseases is unleashed by the transplantation of hematopoietic stem cells.



Today it is known that, besides the bone marrow, placenta and umbilical cord, there are other places in the human body where there are stem cells. The human brain contains very versatile progenitors capable of differentiating into the various types of nerve cells. Muscle, liver and adipose tissue also have several types of stem cells.

If we focus on the nonhematopoietic stem cells that can be transplanted (the other 2% we mentioned earlier), there are already three procedures for the isolation and culture of stem cells that can be considered as common and well-established. There are several Spanish hospitals that carry out keratinocytes grafts in the case of major burns and chondrocytes (cartilage-producing cells) for articular lesions. In addition, so-called cornea resurfacing with limbal cells, amniotic membrane, or epithelial cells of various mucosa is also a common procedure in the treatment of ocular lesions with fairly satisfactory outcomes.<sup>24</sup>

A stem cell of a certain tissue can, in theory, become mature cells of another tissue. This is known as transdifferentiation and occurs with greater probability when the progenitor cell has more plasticity. Experimentally, stem cells (especially bone marrow or adipose tissue mesenchymal cells) have been used for the regeneration of soft tissue, such as the case of the treatment of fistulas occurring in Crohn disease, to accelerate the remodelling of bone fractures, or to regenerate bone tissue in cystic cavities or after tumour removal.

In the same way, myocardial cell therapy, either using hematopoietic precursors or myoblasts, despite not having been shown to be fully effective and the mechanism being poorly understood, has raised great hopes for many patients suffering from cardiac insufficiency.

Embryonic stem cells have become, with or without justification, the great hope of many millions of patients across the world. On few occasions in the history of medicine have expectations been raised so high. In theory, the possibility to have unlimited quantities of these cells, to culture them, and to make them differentiate into cells of the liver, nervous system or heart or even insulin-producing Beta pancreatic cells would in fact become the ideal solution for the treatment of millions of patients.

## CONCLUSION

These therapeutic approaches if effective, may eliminate the need for many transplants carried out these days. However, there are many problems to solve and many frontiers to cross. To mention an important one, we still do not know how to make a human embryonic cell differentiate into the specific cell-type we want, let alone into a complex and structured organ. We simply do not know the signals that make this possible. Along the same lines, we do not yet know how to stop their proliferation, with the theoretically possible consequence of tumour generation which currently makes their clinical use nonviable.

Moreover, there are other problems. Everyday it seems more evident that the many times it has been announced as fact that it is possible to create cells with certain characteristics (such as Beta pancreatic cells) does not imply that in *in vivo* experiments (in animals or in humans) these cells are going to behave like the real beta-producing cells.

It is nevertheless understood that all these possibilities, and many other barely conceived, have raised great expectations both in the scientific community and general population. These are the new frontiers of medicine, many of which will be mastered in the future. It is quite plausible that what organ transplantation has represented in the 20th Century, bringing down taboos and saving hundreds of thousands of lives, is going to be replaced by stem cell therapy in the 21st Century.

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## CHAPTER 2

# ORGAN TRANSPLANTATION IN THE 21th CENTURY

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**Abstract:** Mankind has always been interested in investigating and searching for solutions regarding the body deterioration due to factors such as disease, damage caused by trauma, toxins or radiation or just the process of ageing. Here, we summarize the history of scientific advances in solid organ transplantation, in the areas strictly linked to transplantation. The period between the end of the 19th and beginning of the 20th century has been called by some authors the “Era of allografting”. This was a muddled period with many studies and publications on very diverse transplants, from Kocher’s (Nobel Prize in 1909) who transplanted thyroid extracts, to Brown-Sequard who tried to rejuvenate people by using grafts of guinea pig testicle extracts. In the midst of the 20th century, Sir Medawar pointed out that the rejection of transplant organs by the recipient body was mediated by an immunological reaction, which should be modified. Since then, there has been an open period of discovery of new immunosuppressive drugs which have revolutionised the outcomes of solid organ transplantations. New challenges have appeared over the last few years, these efforts have focused on the search to extend graft durability and with it recipient patient survival times, as well as improve their quality of life.

## INTRODUCTION

Nowadays there is tendency to think that everything has appeared yesterday, that only what has been recently invented is valid. This myth is especially dominant in the field of organ transplantation. The truth is that mankind has always been interested in investigating and searching for solutions regarding the body deterioration due to factors such as disease, damage caused by trauma, toxins or radiation or just the process of ageing.

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It should be noted that this chapter refers mainly to the history of scientific advances in solid organ transplantation, in the areas strictly linked to transplantation. It does not pretend to deal with achievements and conflicts in legal, social, economic, ethical, organizational and other fields, nor does it mention important angles such as the introduction of dialysis as a support technique for renal transplants, organ conservation in deceased donors, mechanical circulatory assistance, cell therapy, the use of biomaterials, organogenesis, stem cells, and organ retrieval from deceased donors.

Table 1 shows a summary of the main milestones reached in the course of the story we are going to recount.

## THE FIRST TRANSPLANTS

The first record of a transplant dates from 16th century BC, when the surgeon Sushruta carried out tissue transplants in patients in India. Specifically, he developed a technique for nose repair using forearm skin,<sup>1</sup> which was used again later by Gaspar Tagliacozzi in the 16th Century. John Hunter carried out skin, testicle and ovary grafts in the 18th Century, made reference to Tagliacozzi's technique, and coined the term transplant.<sup>2</sup>

Jacobus de Voragine, Archbishop of Genova in the 13th Century, recounts in his "Golden Legend of Lives of the Saints", how a man in charge of looking after the Parisian temple devoted to Saint Cosme and Saint Damian, who was severely suffering from a tumour in his leg, woke up one morning without pain, and with a leg that had been transplanted by the saints, recovered from the body of an Ethiopian who had died the previous day (Fig. 1A).<sup>2</sup>

Beyond mythical stories, in the 18th Century, Giuseppe Baronio carried out skin grafts successfully between sheep according to Paul Bert, disciple of Claude Bernard, in 1863.<sup>3</sup> This author carried out several skin grafts successfully in Necker Hospital in Paris. The first scientific experiments in organ transplantation in dogs were performed in Vienna in 1902, by Emerich Ullmann and Decastello.<sup>4</sup> The École Lyonnaise with Mathieu Jabulay and Alexis Carrel<sup>5</sup> (Nobel Prize in 1912) optimised the vascular anastomosis technique, using dog carcasses (Fig. 1B). Alexis Carrel later emigrated to USA. There, he continued his work with C. Guthrie at the University of Chicago, where he carried out a heterotopic cardiac transplant, which beat for hours, by suturing the great vessels of the donor's heart to the carotid artery and jugular vein of the receptor dog.<sup>6</sup>

The period between the end of the 19th and beginning of the 20th century has been called by some authors the "Era of allografting". This was a muddled period with many studies and publications on very diverse transplants, from Kocher's (Nobel Prize in 1909) who transplanted thyroid extracts, to Brown-Sequard who tried to rejuvenate people by using grafts of guinea pig testicle extracts.

## The Renal Transplant

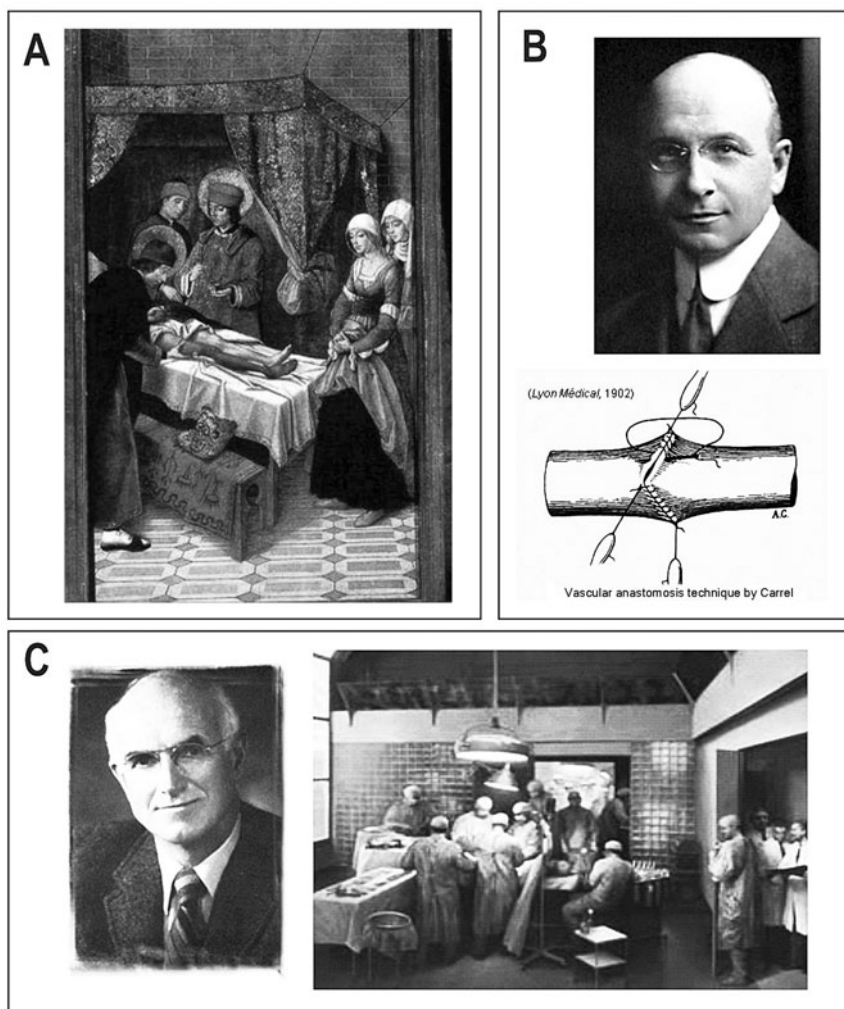
In Russia 1933 Yury Y Voronoy<sup>5</sup> carried out the first renal transplant from a 60-year-old deceased male donor to a young woman in uremic coma due to mercury poisoning. For around two days after the transplant, the graft inserted in the thigh functioned poorly and then the patient suddenly died. The same researcher, Voronoy reported in 1949, having performed five kidney transplants from dead donors, but this time with no success.

In the nineteen fifties, kidney transplants in dogs start to be performed in an organised way, but there was no treatment for transplant rejection at that time. Only at the end

Table 1. History of solid organ transplantation

EXPERIMENTAL ERA (1947-1961)					AZATHIOPRINE ERA (1962-1983)			CsA ERA (1984-1995)	CURRENT ERA (1995- )
1905 A. Carrel y C.C. Guthrie University of Chicago: heart transplant as a experimental surgical possibility	1947 Boston. 1 <sup>st</sup> kidney transplant between humans. The recipient survived	1947 Boston. 1 <sup>st</sup> Transplant using immunosuppressants F. D. Moore (Boston). 1 <sup>st</sup> liver transplant T. E. Starzl (Denver) programme in dogs.	1960 Lower and Shumway University Stanford 1 <sup>st</sup> experimental orthotopic heart transplant	1964 Starzl 1 <sup>st</sup> long-term survival of a dog with liver transplant	1967 C. Barnard Groote Schuur Hospital (Cape Town), South Africa 1st heart transplant human to human	Beginning 80 J. Cooper (Toronto) definitive consolidation of single lung transplant	1986 J. Cooper 1 <sup>st</sup> dual lung transplant	1988 Von Hippel 1 <sup>st</sup> corneal graft	
		Goldberg University Maryland 1 <sup>st</sup> orthotopic heart transplant in animals		Hardy heart transplant from a chimpanzee to human at University of Mississippi					
1933 Voronoy. 1 <sup>st</sup> cadaver kidney transplant on a woman	1954 Murray y Merrill: kidney transplant between identical twins (Boston)	1959 Murray. 1 <sup>st</sup> successful kidney transplant between non-identical twins (Boston)	Hamburger repeats this success in Necker Hospital (Paris)	Lillehei 1 <sup>st</sup> experimental transplant of intestine in dogs (Minnesota)	1966 Nelly; Lillehei; Merkel and Idezuki. University of Minnesota 1 <sup>st</sup> pancreas transplant kidney pancreas	1968 The concept of brain death was accepted. Generalised development of liver transplant alternative. Generalisation	1983 Consensus conference Bethesda (Maryland), backed by the NIH declares liver transplant a therapeutic alternative.	1987 Childrens Hospital (Pittsburg) Multiorgan transplant, including complete intestine of dead donor	
				1963 Guy Alexander in Louvain (Belgium) 1 <sup>st</sup> kidney transplant from brain dead beating heart donor.					
				Starzl Hospital Veterans (Denver) 1 <sup>st</sup> liver transplant in humans					
				Hardy in Jackson (Mississippi) 1 <sup>st</sup> lung transplant between humans					

CsA, cyclosporine A; NIH, American National Institutes of Health.



**Figure 1.** A) Picture about the miracle of Saints Cosmas and Damian. According to the “Golden Legend of Lives of the Saints” by Jacques de Voragine, archbishop of Genoa, a man in charge of caring for the Parisian temple dedicated to these saints, suffered enormously because of a tumour in his leg. One morning he woke up without pain and transplanted with a new leg, obtained from an Ethiopian who died the previous day. (From: Pedro Berruguete; 1450-1504; Museo de la Real Colegiata de San Cosme y San Damián de Covarrubias, Burgos, Spain) B) The French doctor Alexis Carrel, Nobel Prize in 1912 for his discovery of vascular anastomosis techniques that allowed the subsequent development of organ transplantation. He also anticipated for first time the possibility of heart transplantation as therapy and the theory of biological incompatibility. (From: Alexis Carrel, [info-dialyse.de](mailto:info-dialyse.de)) C) Joseph E. Murray, American surgeon awarded the Nobel Prize for Medicine in 1990, performed the first successful kidney transplant in humans in 1954. (From: Joseph E. Murray, [nobelprize.org](http://nobelprize.org); and First Kidney Transplant, [thehumanbrainproject.com](http://thehumanbrainproject.com)).

of this decade does the first attempt at treatment appear: According to the work of J.B. Murphy from the Rockefeller Institute,<sup>1</sup> it consisted of applying intensive radiation to the transplant recipient.

**Table 2.** Author, date and location of the first human solid organ transplants

First Solid Organ Transplants between Humans
1954: First kidney transplant, between identical twins carried out by Joseph Murray and John Merrill in Boston, Massachusetts (USA).
1963: First liver transplant, carried out by Thomas E. Starzl in Denver, Colorado (USA).
1963: First lung transplant, carried out by James D. Hardy in Jackson, Mississippi (USA).
1964: First intestine transplant, carried out by David Grant, William Wall and Calvin Stiller at the University of Western Ontario of London, Ontario (Canada).
1966: First pancreas transplant, carried out by Richard C. Lillehei in Minneapolis Minnesota (USA).
1967: First heart transplant, carried out by Dr. Christian Barnard in Cape Town, South Africa.

The first successful human to human kidney transplant took place in Boston in 1947.<sup>7</sup> A young woman who was in deep coma caused by uremia, who had been anuric for 10 days after a septic shock secondary to a difficult abortion, received a kidney from a deceased donor. The implant was inserted in the elbow fold and was maintained warm with the bulb of a lamp. The kidney excreted urine for only one day and then stopped functioning. Despite this, natural diuresis restarted two days later and the patient recovered.

The first kidney transplant by intra-abdominal surgery was performed in Chicago, USA in 1950 on a woman who suffered from kidney polycystosis with impaired renal function. One of the two polycystic kidneys was removed and was replaced by the kidney of a deceased donor. Two months after the operation the kidney was found to be functioning.

After this date, several European and American teams performed various human transplants coming from deceased donors. The kidney transplant carried out in the Necker Hospital in Paris became well known, and has been regularly cited in the literature. It was performed at Christmas 1952 by the team of Michon and Hamburger.<sup>8</sup> A man suffered from severe renal trauma after an accident, and his only remaining kidney stopped functioning. He was transferred to Paris where he received a kidney from his mother which worked for 22 days, before being irreversibly damaged due to acute rejection as expected. This helped to demonstrate the existence of a violent immune response that attacks the foreign organ even among close relatives.

This leads to the first totally successful kidney transplant in 1954 (Table 2). The team composed by Joseph E. Murray (Nobel Prize in 1990) (Fig. 1C) and Hartwell Harrison transplanted a kidney between identical twins in the Peter Bent Brigham Hospital of Boston.<sup>2</sup> The nephrologist in this case and the following transplants was Professor John Merrill.

However, there was no further progress on organ transplantation beyond that between identical twins for some years and there were up to 11 attempts at kidney transplant between non-identical twins, in which the whole recipient body was treated with radiation. Despite this treatment, all resulted in total failure.

Meanwhile, there was great progress in immunological research during the nineteen fifties. The first transplant in which immunosuppressants<sup>7</sup> were used was carried out in Boston in 1958. The kidney survived but the patient died due to infection resulting from immunosuppression. Five years after the success of the renal transplant intervention between the identical twins in 1959, the same team of Murray in Boston, carried out the first successful renal transplant between non-identical twins using radiation.<sup>9</sup>



Some months later, Professor Hamburger and his team repeated this success in the Necker Hospital in Paris. This transplant was followed by four others with long-term survival in two of the cases (15 years in the first case). These were carried out between relatives who were not identical twins. Professor Hamburger's team also used corticoids and set the basis for "applied histocompatibility", maintaining the organs to be transplanted in a good condition.<sup>9</sup>

In parallel another French team, that of Dr. Rene Küss with a long record in the field, described in 1951 a technique for renal transplantation in the iliac fossa, a technique that was used later for the first transparent carried out in Boston. He performed six transplants at the beginning of the sixties, using for the first time a combination of immunosuppressant procedures and drugs: Total body radiation, steroids and 6-mercaptopurine. In this way, he enabled three patients to survive several months, a landmark in the world history of transposition.<sup>10</sup>

The first renal transplant from a "brain dead" patient with a still-beating heart was carried out by Guy Alexandre in Louvain (Belgium) in 1963. The transplant recipient died a month later due to septicemia.

Brain death had already been described by neurophysiologists Mollaret and Goulon<sup>5</sup> as "coma dépassé". In 1964, Alexandre<sup>9</sup> performed the second renal transplant under the same circumstances; however this time the kidney worked for more than six years. That very same year, Hamburger carried out the second renal transplant from a brain-dead donor.

The concept of brain death was accepted for the first time in 1968, which was crucial for the development of organ transplantation from cadavers. In fact, approval of individuals in brain coma becoming organ donors, both from the ethical and technical point of view, opened the doors to the generalised development of renal transplantation.

In 1973, Dr. G.J. Monchik and Dr. P. Russell<sup>9</sup> established a phenomenon that had long since been suspected, that of "graft versus host disease" in rats.

## Liver Transplant

The history of liver transplant is closely linked to that of the surgeon Thomas E. Starzl.<sup>8</sup>

The first liver transplants carried out by Francis D. Moore<sup>11</sup> in Boston and Thomas E. Starzl<sup>9</sup> in Denver in dogs were initiated in 1958 and published in 1960. Moore had carried out 31 transplants of which only 7 animals survived more than 4 days. None of the animals survived more than 12 days due to acute rejection. However, there was no treatment against rejection at that time.

The first live transplant in human was performed, by Dr Starzl in the Veterans Hospital in Denver in 1963, on a three-year-old child who died five hours later. A second transplant was carried out two months later which worked for 22 days, the patient dying of a pulmonary embolism with normal liver function.<sup>8</sup>

In 1964, Starzl achieved the world's first long-term survival of a dog: It lived almost for 12 years, dying of old age.<sup>9</sup> At the time, the animals that were treated with azathioprine only survived between 25 and 50 days, but it was possible to withdraw the immunosuppressant treatment 3 or 4 months after transplantation without any observable rejection. From this practical observation, the notion of immunotolerance emerged.

Demirleau in France, Roy Calne in the United Kingdom and several groups in the United States, with Starzl as the main figure, carried out tens of liver transplants between 1963 and 1967, all of them with survival times less than one year. Dr. Roy Calne at Cambridge University initiated his programme in Europe in 1968, making use of cyclosporine.<sup>9</sup>



As with other nonrenal organ transplants, it was only in the nineteen-eighties, with a consensus conference held in Bethesda (Maryland) in 1983, promoted by NIH, that liver transplant was declared a therapeutic alternative, leading to the spread of the practice worldwide.<sup>8</sup>

### **Lung Transplant**

In 1963, J. Haglin published the results of experiments that demonstrated that complete lung denervation was incompatible with long-term survival in dogs but not in primates.<sup>12</sup> Castaneda was the first researcher to achieve long-term survival times after self transplantation in 23 baboons.<sup>13</sup>

In 1963, James D. Hardy carried out world's first lung transplant between humans, in Jackson (Mississippi) under a serious ethical compromise given that the transplant recipient had the death penalty waived on exchange for agreeing to participate (Table 2). Full lung function lasted for 16 days until the patient died due to an acute on chronic renal failure, a previously existing condition.<sup>8</sup>

Denton Cooley carried out the first heart-lung transplant in 1968 with no success in the Texas Heart Institute. The same year Fritz Derom<sup>14</sup> achieved a 10-month survival time after transplantation of one lung in Louvain (Belgium). As with other transplants, these were abandoned until 1981, when Shumway and Reitz restarted heart-lung transplantations using ciclosporin. Joe Cooper mastered the technique of single-lung and double-lung transplantation at the beginning of the nineteen-eighties and 1986<sup>8</sup> respectively.

### **Intestinal Transplantation**

The first experimental transplant was carried out by Lillehei in dogs in 1959.<sup>15</sup> However, the work was abandoned due to poor results: High rates of morbimortality due to rejection (the intestine is rich in lymphoid tissue) and sepsis (rupture of the intestinal wall).<sup>16</sup>

The first attempts in humans were carried out in the University of Western Ontario (Canada) on two children who received intestine from their mothers with no success. It was not until the nineteen-eighties that this type of transplant began to be performed again, despite having the same problems, especially during the first year (Table 2). Various attempts followed one another, with poor outcomes, so they stopped for some years. In the nineteen-nineties programmes for this type of transplant<sup>8</sup> were established in earnest. A few years earlier, in 1987, a multivisceral transplant that included the complete intestine of a deceased donor was successfully performed in the Childrens Hospital of Pittsburg, although the patient died of lymphoma three months after surgery. In 1988, a team in London (Ontario, Canada) led by David Grant, William Wall and Calvin Stiller carried out a multivisceral transplant (liver and the first part of the intestine), with the recipient surviving and indeed eating for three years.<sup>17</sup>

### **Pancreas Transplantation**

The first pancreas transplants were carried out in combination with those of kidneys in diabetic patients suffering from terminal renal insufficiency, in 1966 in the University of Minnesota by Dr. W. Nelly, R.C. Lillehei, F. Merkel and Y. Idezuki<sup>8</sup> (Table 2). While it only worked for two months, good glyceimic control was achieved without administration of insulin which demonstrate the potential usefulness of this type of transplants.

After this, once the great technical problem of the spillage of pancreatic secretions had been overcome, pancreas transplants have been started again in a more generalised way worldwide with morbimortality rates having decreased considerably with respect to the early transplants.

### **Corneal Transplantation**

The concept of replacing a damaged cornea as a treatment for blindness<sup>1</sup> is attributed to Erasmus Darwin in 1797.

G. Pellier of Kuengsy was probably the first person to carry out a corneal transplantation, while Zirm carried out the first successful corneal graft from the cornea of an enucleated eye in 1905.<sup>1</sup> The first lamellar corneal graft was performed by Von Hippel in 1988.<sup>1</sup>

### **Cardiac Transplantation**

We made reference earlier to the researchers Alexis Carrel and Charles C. Guthrie from the University of Chicago, who considered heart transplant a surgical possibility in 1905.

Later several researchers made various contributions to the surgical procedure such as the application of hypothermia to increase heart viability for hours and mechanical circulatory support of recipients. In this first period, the first cardiac orthotopic transplant was carried out by Dr Goldner in the University of Maryland in 1958, and the experimental animal lived for 117 days. The first programme for experimental cardiac orthotopic transplant was established by Lower and Shumway in Stanford University.

Before carrying out cardiac transplants in humans, James Hardy<sup>18</sup> transplanted the heart of a chimpanzee to a human in the University of Mississippi in 1964; it stopped functioning 90 minutes after the operation, but thereby demonstrated that this type of transplant should be possible between humans.

The first human-to-human transplant was carried out in the Groote Schuur Hospital in Cape Town, South Africa by Professor Christiaan Barnard in 1967, the patient surviving for 18 days<sup>19</sup> (Table 2). The second transplant by Barnard (really the third one, since there had been an unsuccessful attempt in 1968) was the first to cross the barrier of the 12-month survival time. From then, around a hundred transplants are carried out in USA and Europe, with poor results, and it is not until the nineteen-eighties that this programme is relaunched.<sup>8</sup>

When the use of cyclosporine, azathioprine and prednisone became widespread in the nineteen-eighties, the 5-year survival rates increased to 75% and cardiac transplantation became a real treatment option.

### **Xenotransplantation**

The concept of transplanting organs and tissues from animal to humans is ancient. Kidney transplantation from animals was attempted at the beginning of the 20th Century in France and Germany without success, and was then abandoned for a long time for lack of viability. In 1963, Claude Hitchcock of Hennepin County Hospital in Minneapolis carried out a kidney transplant from a mandrill to a woman that functioned for four days. That year Keith Reemtsma of Tulane University carried out a similar experiment with a chimpanzee kidney that worked for 9 months. After this, several transplants of this type were carried out without success.<sup>9</sup> For example, we have already made reference to James Hardy who transplanted the heart of a chimpanzee into a 68-year-old man.

However, one of the groundbreaking landmarks of xenotransplantation in the present era, which led to a new era in this field, was the following. In October of 1995, the director of fundamental research of Imutran (subsidiary of the Swiss Laboratory Novartis), David White, transplanted hearts of transgenic (genetically modified) pigs as well as hearts animals derived from natural reproduction to *Cynomolgus* monkeys. In the first trial he demonstrated that none of the transgenic organs were rejected, and that two of the primate recipients lived for more than 70 days. On the other hand, the monkeys that received hearts from nontransgenic animals died in less than one hour. Since those findings, there has been progress in this field.

Nevertheless, xenotransplantation apart from the difficulties overcoming rejection that is more complicated to control since it involves transplants between species, also presents two other notable problems. First is the ethical issue of the use of primates due to their genetic and other types of proximity to humans. The second issue, which seems insolvable, is the risk of transferring viruses (and less often prions) present in the xenogenic transplant to the human recipient and, hence, the appearance of a new virus by recombination between elements present in the donor's and receptor's genome, which could lead to a new zoonotic disease with unpredictable consequences. This may be considered a high risk too high to be run by human kind as a species.<sup>20</sup> However, such transplants are still being carried out for research purposes. Table 2 shows a summary of the first transplants and some important events in the history of transplantation of each type of solid organ transplant.

## HISTORY OF IMMUNOSUPPRESSION

In the midst of the 20<sup>th</sup> Century, Sir Medawar had already pointed out that the rejection of transplant organs by the recipient body was mediated by an immunological reaction, and if to be avoided, the immune response should be modified. Sir Peter Medawar and his team made another important contribution in 1951: They discovered that hormones that are synthesised in the adrenal cortex (glucocorticoids) and that their mimicking drugs (corticosteroids, steroids and corticoids) were capable of reducing the rejecting immune response, thereby increasing the life of heterologous skin grafts. Since then, there has been a period of discovery of new immunosuppressive drugs which have revolutionised the outcomes of solid organ transplantations, giving rise to the various "eras" discussed below (Table 3).

### Experimental Era

This spans from 1947 to 1962 and involved only kidney transplantation. The outcomes were very poor and the main obstacle to progress being the absence of adequate immunosuppressive therapy. As mentioned earlier, in the nineteen-fifties adverse immune response was avoided by transplanting between identical twins (Murray 1954). During this period, the treatment for rejection using total or partial irradiation of the graft and spleen was initiated. However the doses used, 1,000 rads, common for radiotherapy, were too high and animals died due to infections. When lower doses were applied, the recipients survived but the transplanted organ was rejected.

Other techniques tried were splenectomy, thymectomy (both still used in certain experimental protocols in combination with other techniques and immunosuppressive drugs) and thoracic duct drainage.

**Table 3.** History of the discovery of the main immunosuppressive drugs used in the prevention and treatment of solid organ transplantation

• 1950s	• Steroids
• 1960s	• Azathioprine
	• Polyclonal antibodies
• 1970s	• Cyclosporine A
	• Monoclonal antibodies OKT <sub>3</sub>
• 1980s	• Tacrolimus
	• Cyclosporine microemulsion
• 1990s	• Mycophenolate mofetil
	• Sirolimus
	• Thymoglobulin
• 2000s	• Other monoclonal antibodies
	• Mycophenolate sodium (enteric coating)
	• Everolimus
	• Slow released tacrolimus

### Era of Azathioprine

This spans more or less between 1962 and 1983 and also exclusively involved kidney transplantation. It was characterized by the search for adequate immunosuppression, since until then, acute rejection rates corresponded to around 90 and 100% of cases.

Dr. David Hume, and his collaborators C.Zukoski and H.M. Lee, started some experiments with a new drug, 6-mercaptopurine, aiming to replace the use of radiation. The first results were made public in 1960.<sup>22</sup> These findings, together with the experiments on rat skin and renal transplantation in dog by Sir Roy Calne<sup>23</sup> lead to positive and promising results.

Back in 1952, 6-mercaptopurine and derived compounds had been synthesised by Dr George Hitchings and Gertrude Elion in Burroughs Wellcome's laboratories in New York. Then, in 1959, just one year before being used in the transplant, Dr. Robert Schwartz and Dr. William Dameshek, at Tufts University in Boston demonstrated that it was involved in the inhibition of the immune response in rats.<sup>24</sup> A few months later Imuran® (called Imurel® in Spain), a compound derived from 6-mercaptopurine, started to be used.

At the beginning of the nineteen-sixties, Byron Walkman at the Yale University, Michael Woodruff at the University of Edinburgh and Anthony Monaco and Paul Russell at Harvard University used, for the first time, antilymphocyte serums for treating skin experimental transplants. Since then, several initiatives have published, with that of Peter Medawar being especially notable.<sup>9</sup> Experiments involved immunising rabbits against guinea pig, mouse and rat lymphocytes. Subsequently, serum from an immunised rabbit was intraperitoneally injected into the recipient receiving the skin graft from these animals. In this way, the transplants were maintained stable much longer. However, the main difficulty was to convince the doctors, already familiar with the use of azathioprine and steroids, to inject antilymphocyte serums to the patients receiving the organs. In summer 1966, Dr. Starzl selected the first patients in the world

to be treated with antilymphocyte globulins prior to transplantation: They came from Colorado and Denver VA General Hospitals.<sup>9</sup> With this success, resounding according to Dr. Starz, a new era of rejection treatment commenced, combining azathioprine and steroids (at lower doses than previously) and antilymphocyte globulins. Later, Ben Cosini using the hybridoma technique cloned the immortalized cells that produced the OKT3 monoclonal antibody. This was used for the first time in 1980 in Massachusetts General Hospital.

On the other hand, Carl Landsteiner in his 1931 Nobel Prize acceptance speech suggested the possibility of investigating tissues that are similar to blood: The groups that he had described earlier. In London, Gorer, demonstrated the existence in mice of what Snell, working in the USA, named the histocompatibility<sup>2</sup> system. In 1952, Jean Dausset, who was awarded the Nobel Prize in 1980, Van Rood and Rose Payne, independently detected and identified antibodies in serum, in Dausset's case from patients who had undergone blood transfusions, and in the other two cases from multipara women. Later, Dr Paul Terasaki developed the corresponding identification tests.

In fact, at the beginning of the nineteen-eighties, Paul Terasaki developed a technique to detect histocompatibility antigens using cytotoxic antibodies at the same time as Jean Dausset.<sup>25</sup> Both of them realised that there was a positive correlation between the degree of compatibility donor-recipient and the outcome of the transplants. Dr Dausset had previously described, in 1958, the first leukocyte antigen of the mouse MHC (Major Histocompatibility Complex), that was to be found to correspond to the HLA (Human Leucocyte Antigen).

### **Era of Cyclosporine**

This spans from 1983 to 1995. There is a substantial improvements in outcomes, since the main problem at the time, acute rejection had been reduced by 50-60% in all renal transplant cases. However, the new medication turned out to have considerable nephrotoxicity. Despite this, it was soon confirmed that they enable the graft to remain stable for longer.

Indeed in 1971, a drug called cyclosporine was discovered in the United Kingdom in Sandoz's pharmaceutical laboratory. Jean Borel demonstrated that it partially suppressed rejection of rat skin grafts.<sup>26</sup> Sir Roy Calne start to use it and defined it as "sufficiently" potent and with low enough toxicity for it to be an interesting immunosuppressant for clinical experimentation in recipients of organ transplants.<sup>27</sup> This author started trials in renal transplant patients at the University of Cambridge in 1978.

On the other hand, in 1980 Dr. Starzl described the progress of the first liver transplants using ciclosporin, although the monitoring spanned from only 1 to 4 months. However the eight patients studied were in good health; this type of transplant had never before been seen to cause so few problems. Therefore the achievements in renal and liver transplantation confirmed that a new era had started, giving rise to the generalisation of liver transplantation worldwide.<sup>28</sup>

At the end of 1981, it was already known that the combined treatment with ciclosporin and prednisone was superior to any other combination of immunosuppressants. Nevertheless, political and administrative problems did not allow solid transplants other than renal to be classified as anything more than experimental. Already on the market, ciclosporin was launched in the United States in 1983 and subsequently in other countries.

**Table 4.** List of antibodies against T or B cells, or both, currently used in prevention and therapy and in Phase II and III clinical trials

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Depleting antibodies (against T, B cells or both):
Polyclonal antibodies: Horse and rabbit antithymocyte globulin
Murine anti-CD3 monoclonal antibodies
Humanised anti-CD 52 monoclonal antibody (Alemtuzumab)
B-cell anti-CD20 monoclonal antibody (Rituximab)
Nondepleting antibodies:
Humanised and chimeric anti-CD25 monoclonal antibodies (Daclizumab, Basiliximab)

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## The Current Era

From 1995, new drugs are discovered with spectacular results, especially in the short-term, the proportion of acute rejection decreasing below 20% in renal transplants. In the mid nineteen-eighties, the University of Tsukuba of Japan, under the sponsorship of Fujisawa Pharmaceutical Corporation, discovered an immunosuppressant drug that they called FR 900506 and later FK-506 or tacrolimus. This calcineurin inhibitor drug was demonstrated to be a more potent immunosuppressant than ciclosporin with similar overall toxicity.

At the beginning of the nineties, Mycophenolate Mophetil (MMF), the 2-morpholinoethyl ester of mycophenolic acid (MPA), started to be used. MPA is a potent, selective, noncompetitive and reversible inhibitor of inosinemonophosphatedehydrogenase; it inhibits therefore, the de novo synthesis of guanosine nucleotide, such that it cannot incorporate into DNA. Given that T and B lymphocytes greatly depend upon the de novo synthesis of purines, unlike some other type of cells that are able to use purine recycling pathways for their proliferation. MPA has a more potent cytostatic effect in lymphocytes and in other cells. The combination of both drugs, tacrolimus and MMF, with steroids is currently the most used immunosuppressant therapy, and in principle the most effective one.<sup>29</sup> It spectacularly improved outcomes: Acute rejection rates fell below 20%, nephrotoxicity decreased, and slight improvement was seen in the long-term stability of grafts. Indeed, this improvement might have been even greater if transplantation had continued to involve mostly young donors and recipients.

Around the beginning of this century, two anti-CD25 monoclonal antibodies began to be used: Basiliximab and Daclizumab, both chimeric and humanized antibodies, allowing selective immunosuppression of T lymphocytes, without causing cytokine release syndrome (as happens with polyclonal antibodies that are still used such as horse and rabbit anti-thymocyte globulins). It does not cause over-immunosuppression, therefore reducing the incidence of infectious and lymphoproliferative disorders and a clear decrease in acute rejection is achieved. Table 4 shows a list of antibodies that are currently used in clinical practice and in Phase II and III clinical trials.<sup>30</sup>

Proliferation signal inhibitors inhibit m-TOR, a kinase involved in cell growth and proliferation, thus stopping the cycle. Although several have been discovered, the most used in practice are Rapamycin (also called Sirolimus) and Everolimus. These drugs, apart from their immunosuppressive action, their lack of nephrotoxicity, and the fact that they have antiproliferative properties (anti-angiogenic, anti-arteriosclerosis and antitumoral), become even more relevant due to the more advanced age of current transplant recipients and the chronic nephropathy associated with kidney transplants.



## CONCLUSION

New challenges have appeared over the last few years, at the beginning of the 21<sup>st</sup> Century. Short-term outcomes have considerably improved. However medium- and long-term results have not improved as much for various reasons: Nephrotoxicity of the calcineurin inhibitor drugs, increased vascular risk due to the appearance or worsening of diabetes mellitus, high blood pressure, and hyperlipidemias among other factors. So, efforts have focused on the search to extend graft durability and with it recipient patient survival times, as well as improve in their quality of life.

The tendencies could be summarized as follows:

1. The search for genomic, proteomic and/or metabolomic biomarkers for early detection of so-called chronic transplant nephropathy, which can appear as early as three months after renal transplantation, often leading to graft failure, and also occurs with other transplants.
2. The search for new protocols to avoid the use of steroids altogether immediately after transplant, or that aim to withdraw or minimize their use in order to avoid the secondary effects they entail for cardiovascular risk, as well as the well their known harmful effects on bone, the increased susceptibility to infection, etc.
3. The search for protocols that avoid the use of anticalcineurin inhibitors immediately after transplant, or a short time later, or aiming to withdraw or minimize their use in order to reduce nephrotoxicity and to improve graft and recipient patient survival using mainly proliferation signal/mTOR inhibitors.
4. The development of immunotolerance, meaning, in a strict sense, the acceptance of the graft by the recipient without immunosuppressive medication. The objective is to reprogramme the immune system in specific way so that any damaging immune response can be stopped, but without jeopardizing the body's normal response to disease. Along these lines, various initiatives have been launched, such as that of the Division of Allergy, Immunology and Transplantation (DAIT) at the National Institute of Allergy and Infectious Diseases (NIAID), NIH in the United States, which has established, co-ordinates and manages the Collaborative Network for Clinical Research on Immune Tolerance. The objectives of this collaborative network are to design a long-term research agenda to accelerate the application of research into tolerance for the treatment of many immune-based diseases and solid organ, cell and tissue rejection. Specifically, clinical trials are being designed and carried out to evaluate the safety, toxicity and efficacy of promising tolerance induction strategies, as well as studies on the mechanisms of tolerance induction, maintenance and loss. Another initiative is Riset (Reprogramming the Immune System for Establishment of Tolerance), a multinational European project, financed by the European Commission under the Sixth Framework Programme, that is to focus on the translation of these advances in research into clinical practice and industrial development.
5. The search for quality of life and adherence and compliance to immunosuppressive therapy: In relation to this, we have seen the appearance of new immunosuppressive drugs such as enteric-coated Sodium Mycophenolate to avoid adverse gastrointestinal effects, so common to date, and extended-release tacrolimus to reduce the number of doses.

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## CHAPTER 3

# IMMUNOLOGY AND THE CHALLENGE OF TRANSPLANTATION

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**Abstract:** Transplantation of tissues or organs between individuals who are not genetically related often leads to rejection by the recipient. The human genes responsible for this process are located on the short arm of the chromosome 6 and are called Major Histocompatibility Complex (MHC). Six main loci have been identified in the human MHC: HLA-A, HLA-B and HLA-C belong to the HLA class I, while HLA-DP, HLA-DQ and HLA-DR belong to HLA class II. The physiological function of MHC molecules is to present peptides to the T cells. Indeed, they are integral components of the ligands that recognise most T cells, since the receptor of the T cell (TCR) has specificity for complexes of foreign antigenic peptides, and self-MHC molecules. Thus the proteins of the MHC are responsible for the body being able to distinguish between its own and foreign cells, known as self-tolerance and consequently are the proteins which determine the evolution of transplants. The special case of foreign MHC antigen recognition is known as allorecognition and consists of the capacity of T cells to recognise peptide/MHC complexes with which they have not been in contact during the process of maturation in the thymus. There are two mechanisms of allorecognition, direct and indirect; both can lead to rejection of the transplant. Direct recognition prevails during the first few weeks or months after transplantation, and is caused by the APCs of the donor. These cells start disappearing from the transplanted organ and indirect recognition becomes important. There is evidence that the indirect pathway is sufficient to mediate both acute and chronic rejection. In this chapter we will describe fundamental aspects of the MHC system, as well as, specifically, its involvement in the allogenic response of the immune system against organ transplants.

## INTRODUCTION

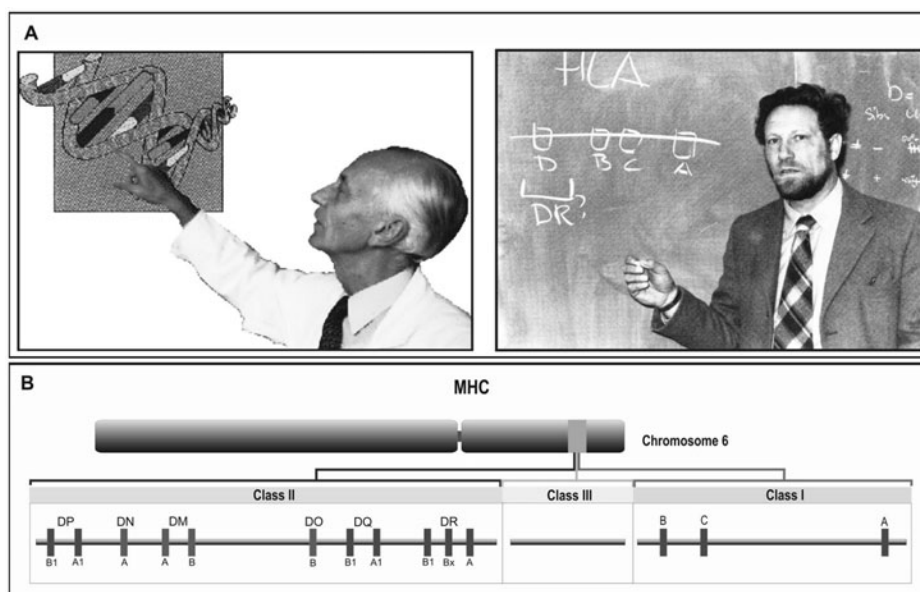
Transplantation of tissues or organs between individuals who are not genetically related often leads to rejection by the recipient. However, when carried out between genetically identical individuals, this does not occur. These types of observations were noted at the beginning of the nineteen forties by Peter Medawar when he performed autologous and allogeneic transplants of human skin.<sup>1</sup> Transplant antigens were described by Snell at the end of the same decade, when he observed rejection of skin grafts and tumours between nongenetically identical individuals.<sup>2</sup> When characterising the genes responsible for this process, he identified just one locus, which he called H2. This locus is a complex of murine genes on chromosome 17, was characterised as Major Histocompatibility Complex (MHC).

The characterisation of MHC in humans occurred later than in mice. In 1953, Jean Dausset found that, after many blood transfusions, individuals presented serum antibodies capable of inducing leukocyte adhesion.<sup>3</sup> Some years later, in 1957, Rose Payne described the presence of these leukoagglutinins in the serum of all patients who have received multiple blood transfusions and in multipara women.<sup>4</sup> He deduced that, in a similar way to in multiple transfusions, multiple pregnancies result in immunisation, in this case, of the mother against the father's leukocyte antigens. In the same year, in The Netherlands, Jon van Rood reached similar conclusions when he saw a patient who, after previous unproblematic pregnancies, developed a severe febrile reaction when pregnant for the seventh time.<sup>5</sup> All these studies demonstrated the need for a better understanding of the biology of these highly immunogenic proteins and, to this end, a series of techniques such as leukoagglutination, cytotoxicity and complement fixation began to be developed. Using antisera reactivity patterns, Dausset grouped these antigens as "MAC" and Van Rood as "H4a/b", while Payne called them "LA-1" and "LA-2". The first International Histocompatibility meeting, organised by D. Bernard Amos in 1964, led to the acceptance that all genes coding these human antigens were located in the same locus (Fig. 1A) and in 1965 a unified nomenclature, of HLA (Human Leukocyte Antigens), was agreed. Although the role of MHC polymorphism in organ rejection was not defined until much later, an important contribution was made by the research of Patel and Terasaki,<sup>6</sup> who suggested in 1969 that recipient antibodies against MHC antigens were associated with early or immediate rejection of grafts.

The main functions of the T-lymphocytes include defence against intracellular pathogens and activation of other cells within the immune system. These functions require interaction of the T-lymphocytes with other cells, since they are only capable of recognising antigens displayed on the cell surface.

The physiological function of MHC molecules is to present peptides to the T cells. Indeed, they are integral components of the ligands that recognise most T cells, since the receptor of the T cell (TCR) has specificity for complexes of foreign antigenic peptides and self-MHC molecules.<sup>7</sup> There are two main types of MHC molecules, MHC class I and II. MHC class I molecules present cytoplasmic peptides to T CD8<sup>+</sup> lymphocytes, while class II present extracellular peptides to T CD4<sup>+</sup> lymphocytes. These proteins are also responsible for the body being able to distinguish between its own ("self") and foreign cells, known as self-tolerance, and consequently are the proteins which determine the evolution of transplants.

In this chapter we will describe fundamental aspects of the MHC system, as well as, specifically, its involvement in the allogenic response of the immune system against organ transplants.



**Figure 1.** A) The photographs show two of the pioneers in the discovery of the human MHC. The image on the left corresponds to Dr. Jean Dausset, Nobel Prize in 1980 and the right to the Dutch researcher Dr. Jon van Rood. B) Genetic map of the human MHC region, located on the short arm of chromosome 6.

## GENETICS OF MHC

Six main loci have been identified in the human MHC: HLA-A, HLA-B and HLA-C belong to the HLA class I, while HLA-DP, HLA-DQ and HLA-DR belong to HLA class II. There are also other less important loci which are much less polymorphic and that code for the nonclassical MHC molecules. These are E, F, G and H in class I and DM, DN and DO in class II<sup>8,9</sup> (Fig. 1B). A large number of alleles have been identified in each locus and this number is rapidly increasing. At this moment (April, 2010), the total was 965 A, 1543 B and 762 DRB1 alleles.<sup>10</sup> Currently several websites provide up to date information about genetic maps for this region. These sites, such as that run by the Anthony Nolan Trust “International Immunogenic Project” ([www.ebi.ac.uk/imgt/hla/](http://www.ebi.ac.uk/imgt/hla/)), are easy to access and in common use.

In humans, the MHC is located on the short arm of the chromosome 6 and a gene on chromosome 15 codes for  $\beta 2$ -microglobulin. Most proteins involved in the processing of antigenic proteins are coded by genes in this chromosome region. MHC class I genes are located in the most telomeric region and those of MHC class II in the centrometric region. Within the class II loci, there are other genes that code for proteins involved in antigen processing, such as TAP (transporter associated with antigen processing) and certain proteasome subunits. The genes coding for the MHC region are very close together, with recombination rates during meiosis of 3% between the most far apart regions. This means that, in general the alleles that code for each variant are transmitted together. Each particular combination of alleles of genes present in a chromosome is known as haplotype.

The MHC system has a high degree of **polymorphism**. Genetic polymorphism is the presence of more than one allele at the same locus, and a locus is considered to be polymorphic if the less common allele appears in more than 1% of the individuals of a population. Many human proteins are polymorphic, but most of them have only few variants or allotypes and generally one or two of these are dominant. However, in the case of MHC proteins, there are hundreds of numbered variants and all of them are relatively common in the population. The differences between the variants are found in the area of union to the peptide, which means that each variant can bind a range of different peptides, even those derived from the same protein. In turn, this means that the type of peptide that each individual carries depends on their MHC phenotype, and therefore varies from one person to another. Also, the expression of these molecules is codominant, both of the inherited alleles being expressed and maximising the number of MHC molecules capable of binding peptides for their presentation to the T cells. Accordingly, it is very unlikely that two individuals selected at random have the same allotype.

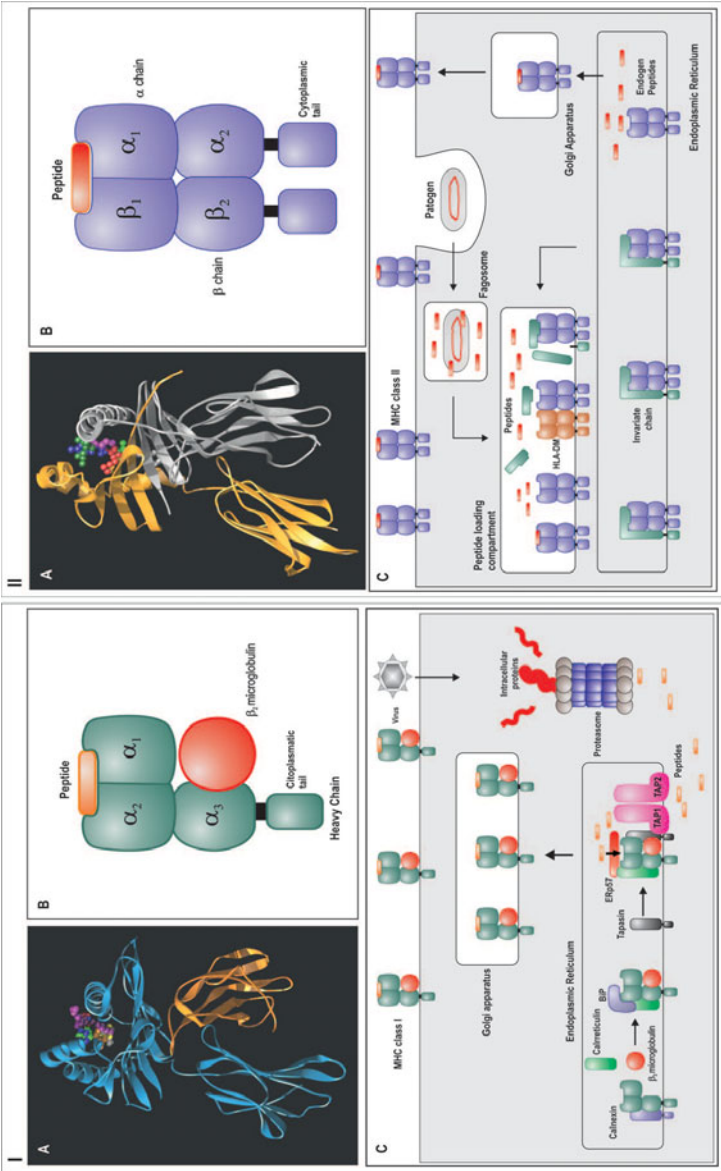
All this variety is very useful for fighting against microorganisms, however it is a barrier to tissue and organ transplantation, and it is what gives the name to the MHC molecules. The fact that every individual has different surface MHC molecules means that grafts carry on their surface foreign antigens that can be recognised by the recipient and are destroyed in the rejection process. For this reason, when carrying out a transplant, the aim is to match two individuals, recipient and donor, in which MHC molecules are as similar as possible. In this way, the risk of rejection is reduced, consequently increasing graft survival.

## STRUCTURE OF HLA MOLECULES

### MHC Class I Molecules

MHC class I molecules consists of a 43 kDa  $\alpha$  transmembrane polypeptide chain, linked by noncovalent bonds to a small extracellular 15 kDa protein known as  $\beta$ 2-microglobulin<sup>11,12</sup> (Fig. 2A). The  $\alpha$  chain is composed of three extracellular domains ( $\alpha$ 1,  $\alpha$ 2 and  $\alpha$ 3), a transmembrane region and a cytoplasmic domain.  $\beta$ 2-microglobulin is linked by noncovalent bonds to the  $\alpha$ 3 domain, which has a structure that is relatively conserved that interacts with CD8 molecules, found on the surface of T-lymphocytes. The  $\alpha$ 1 and  $\alpha$ 2 domains are most external and fold to form a groove for peptides presented by the molecule. This area is called peptide binding groove and for steric reasons, due to the fact that both ends of this groove are closed, it can only recognise peptides of 8-10 amino acids. This is also the most polymorphic region, with variations both in the electrostatic charge and in the size of the groove, and determines the range of peptides presented by the various alleles of MHC class I molecules. The  $\alpha$ 3 segment of the chain is an immunoglobulin type domain, whose amino acid sequence is conserved in all the MHC class I molecules. This is the region that binds to CD8 molecules. In the carboxyl-terminal of this region there is a segment of about 25 hydrophobic amino acids that crosses the lipid bilayer of the plasma membrane. The next 30 residues in the cytoplasm interact with the polar groups of phospholipids on the cytoplasmic side of the membrane anchoring the molecule.

The light chain of the MHC class I molecules is coded by a gene located outside the MHC region. When it was discovered, it was found to be identical to a protein previously



**Figure 2.** CLASS I: A) Three-dimensional structure by X-ray crystallography of a MHC class I molecule associated with  $\beta_2$ -microglobulin. C) Processing and presentation of peptides associated with MHC class I.  $\alpha$  chains of MHC class I molecules and  $\beta_2$ -microglobulin are synthesized in the endoplasmic reticulum (ER). These molecules bind peptides from proteins present in the cytosol that are degraded in the proteasome and transported to the ER lumen by TAP proteins. CLASS II: A) Three-dimensional structure of a MHC class II molecule obtained by X-ray crystallography. B) Schematic figure of a MHC class II molecule composed of two polymorphic chains,  $\alpha$  and  $\beta$ , bond by noncovalent way. C) Processing and presentation of peptides associated with MHC class II. Exogenous antigenic proteins undergo endocytosis and degradation in lysosomes. The  $\alpha$  and  $\beta$  chains of MHC class II molecules are synthesized and linked in the ER. From this they are transported to the cell surface by exocytic vesicles which are merged with other carrying peptides. Occasionally, peptides from cytoplasmic and membrane proteins can enter class II processing.

identified in urine. This chain is known as  $\beta$ 2-microglobulin due to its electrophoretic mobility ( $\beta$ 2), its size (micro) and its solubility (globulin). Like the  $\alpha$ 3 domain, with which linked by noncovalent bonds, it is structurally homologous to an immunoglobulin domain, and its structure is identical in all class I molecules.

The stable expression of the cell-surface MHC class I molecules requires the presence of a peptide anchored in the groove. The reason for this is that the interaction between the  $\alpha$  chain and  $\beta$ 2-microglobulin is stabilised with the binding of the peptide to the groove formed by  $\alpha$ 1 and  $\alpha$ 2, while the interaction between  $\beta$ 2-microglobulin and the  $\alpha$  chain stabilises the binding of the peptide to the molecule. In general, only MHC molecules loaded with peptides are expressed on the surface of the cell.

MHC class I molecules are expressed on the surface of all the nucleated cells of the body, with the exception of the foetal trophoblast. Individuals express six different types of class I molecules on each cell, each of them containing the  $\alpha$  chains derived from the two alleles of HLA-A, HLA-B and HLA-C inherited from each parent.

### MHC Class II Molecules

The three-dimensional structure of class II molecules is very similar to those of class I despite being composed of two equal size transmembrane chains linked by noncovalent bonds<sup>13</sup> (Fig. 2B). These are called  $\alpha$  and  $\beta$  and are coded by the polymorphic genes of the MHC. The  $\alpha$ 1 and  $\beta$ 1 amino terminal segments interact with one another to form the groove for binding the peptide, similar to the class I molecule and contain the most polymorphic residues. In human class II molecules, most of the polymorphisms are located in the  $\beta$  chain. In contrast to class I molecules, the ends of the groove are open, allowing peptides of 30 or more residues to be loaded. The  $\alpha$ 2 and  $\beta$ 2 segments are folded into an Ig-type domain, and several alleles that code for them are nonpolymorphic. A loop in the  $\beta$ 2 segment is the binding site for CD4 molecules. In general, a chain of a MHC locus (for example, DR) will couple with a  $\beta$  chain of the same locus and, also though less often, with a  $\beta$  chain of a different locus (such as, in this case, DQ or DP).

The carboxyl terminal of the  $\alpha$ 2 and  $\beta$ 2 segments are still connected in short regions of 25 hydrophobic amino acids that form the transmembrane regions, followed by hydrophilic cytoplasmic tails of basic amino acids. One nonpolymorphic polypeptide, known the invariant chain (Ii), binds to recently synthesised class II molecules, so that the complete molecule is a heterotrimer composed of  $\alpha$ ,  $\beta$  and Ii chains.

MHC class II molecules are not as widely expressed as class I. They are mainly found in what it is known as professional antigen presenting cells (APCs): Dendritic cells, macrophages and B lymphocytes.

Six class II alleles are inherited, three from each parent (DQ, DP and DR). However heterologous associations can be formed between  $\alpha$  and  $\beta$  chains of each of the chromosomes, for example DQ $\alpha$  of one chromosome with DQ $\beta$  of another. In this way, the number of class II molecules in heterozygotic individuals is more than the six inherited alleles.



## MOLECULES OTHER THAN THE MHC INDUCTOR OF ALLORESPONSES

### The MIC System (MHC Class I Chain-Related Genes)

The MIC system is composed of two families of highly polymorphic genes related to MHC class I molecules, known as MICA and MICB, and that are expressed as a response to cellular stress.<sup>14</sup> These genes are located on chromosome 6, close to HLA-B locus, and code for cell surface proteins that are not associated with  $\beta$ 2-microglobulina and that do not bind peptides. One of their receptors is the molecule NKG2D, and this was originally identified on the surface of NK cells,  $\gamma\delta$  and CD8<sup>+</sup> T-lymphocytes. The MICA molecule could be a target for specific antibodies and T cells in the transplantation of solid organs or in graft versus host disease. In fact, in studies carried out in individuals receiving kidney and heart transplants, there is substantial evidence that the anti-MICA antibodies are associated with transplant failure.

### Minor Histocompatibility Antigens

These are another group of polymorphic proteins, distinct from the MHC system, which have been proven to cause rejection in transplants. Peptides of these proteins are presented to MHC class I- and class-II restricted T cells. The number of possible minor histocompatibility antigens in transplants between nonrelated genetically related individuals is very high. However this type of reaction seems to be restricted to a few immunodominant epitopes.<sup>15</sup> The molecular basis for this process is unknown, but it has been described that the magnitude of the cytotoxic response that they cause, depends on both the duration of the presentation and the avidity of T-cell recognition. The number of T cells responding to these antigens in individuals who have not received a transplant is very low, and it is only possible to measure this in vitro after in vivo vaccination or after repeated stimulation. In these cases, it has been observed that the cells that respond to these antigens are usually CD8<sup>+</sup> T-lymphocytes, implying that the peptides of the minor histocompatibility molecules bind to their own MHC class I molecules. However, peptides bound to self-MHC class II molecules can also participate in the response to transplants between individuals with identical MHC. The immune response against these antigens can lead to transplant rejection or the appearance of graft versus host disease. Despite the fact that these antigens are known as minor and the number of cells responding against them is low, a single immunodominant antigen of this type may induce graft versus host disease after a bone marrow transplant.

Minor MHC antigens of various genetic and cellular origins have been described. Some of them are encoded by the sex chromosomes, mainly on chromosome Y and they are known as HY. The absence of products of this chromosome in women induces a response to male antigens, especially if they have had male children prior transplantation. Other are encoded by autosomal genes, HA1-HA8, and they have been even found in mitochondrial DNA, known as MTA as they are transmitted by the mother (Maternally transmitted antigens).

## **KIR GENES (NATURAL KILLER CELL IMMUNOGLOBULIN-TYPE RECEPTORS)**

The KIR genetic region is highly variable, both due to the gene content and the allelic polymorphism between individual KIR genes. They are found in a cluster on chromosome 19 in the region called the LRC (leukocyte receptor complex). KIR molecules are expressed on the surface of natural killer (NK) cells and in some subpopulations of T-lymphocytes, depending on whether the cytoplasmic domain that presents the molecule has activating or inhibiting properties. Some KIR receptors have MHC class I molecules as natural ligands. The polymorphism of KIR receptors is especially important in the transplantation of haematopoietic stem cells, in which NK cell alloreactivity has been associated with improved progress of the patient.<sup>16</sup> This alloreactivity derives from the difference between the inhibitory KIR receptors for MHC class I molecules in the NK cells of the donor and the MHC class I ligands expressed in the recipient cells. The function of NK cells is regulated by specific inhibitory KIR receptors for self-MHC class I molecules. The absence of interaction with these receptors leads to the cell lysis, thereby eliminating residual malignant cells in the recipient.

## **ANTIGEN PRESENTATION**

### **Processing and Presentation of Peptides Associated with MHC Class I**

MHC class I molecules present peptides to CD8<sup>+</sup> T-lymphocytes (Fig. 2A). All the nucleated cells of the body, with the exception of placental trophoblasts, can make this type of presentation. Differentiated CD8<sup>+</sup> T-lymphocytes are able to recognise peptide/MHC class I complexes and lyse the cells that exhibit them on their surface. The peptides presented by the MHC class I molecules are obtained from the proteolytic degradation of cytoplasmic proteins.<sup>17,18</sup> Antigenic proteins that are present in the cytoplasm can be the product of a virus or other intracellular pathogens that synthesise their own proteins during their life cycle. Many of an individual's own normal and tumour proteins are also present in the cytosol and can also be processed to be presented by class I molecules. The proteins undergo proteolysis in the cytosol, the main enzyme responsible for this process being a multienzyme complex known as proteasome, although involvement of some others has also been reported. The proteins to be degraded are tagged by covalent attachment of several molecules of a protein known as ubiquitin. The peptides generated in the cytosol are transported by a heterodimeric protein with ATP binding sites, known as TAP (Transporter Associated with antigen Processing), into the lumen of endoplasmic reticulum (ER), where the MHC class I molecules are synthesised. The TAP protein has specificity for a wide range of peptide sizes, but it most efficiently transports peptides of 6 to 30 amino acids with basic or hydrophobic carboxyl terminals, typical characteristics of peptides resulting from proteasome activity. The LMP2 and LMP7 genes, which code for components of the proteasome, and TAP1 and TAP2, polypeptide subunits of TAP, are located in the MHC class II region. On the lumen side of the ER, TAP is bound by a protein known as tapasin, to a recently synthesised MHC class I molecule, already bonded to  $\beta$ 2-microglobulin. When the peptides are transported into the ER using TAP, they bind to the tapasin-associated MHC class I molecule and are released from the tapasin. The complex then leaves the ER moving towards the Golgi apparatus



and the cell surface in exocytic vesicles. In the absence of the peptide, the majority of dimers between the  $\alpha$  chain and  $\beta$ 2-microglobulin are unstable and cannot be efficiently transported outside the ER. Once on the surface of the cell, the peptide/MHC class I complex can be recognised by CD8<sup>+</sup> T cells that are specific for the antigenic peptide. The CD8 molecule plays an essential role through its interaction with the nonpolymorphic regions of the class I molecule, while the function of the MHC class I molecules is to exhibit on the cell membrane a sufficiently wide range of peptides of all the proteins that are being synthesised in the cell, for CD8<sup>+</sup> T-lymphocytes to be able to detect pathogens that are being replicated.

The induction of primary CD8<sup>+</sup> T-lymphocytes responses is particularly complex. Antigen presentation can be produced by a certain type of cell, an infected or tumour cell, which cannot provide the costimulation necessary for lymphocyte stimulation. Apart from the activation signal generated by the interaction between TCR and MHC, effector lymphocytes need to receive additional activation signals, mainly resulting from the binding of CD80 or CD86 to CD28 molecules on the surface of the lymphocyte. It is probable that the APCs (antigen-presenting cells) capture antigens of these cell types and present them to CD8<sup>+</sup> T cells initiating the primary response. This process is known as cross-presentation or cross-priming, that is, one type of cell, APC, may present another cell's antigens and activate specific T cells for that antigen.

### **Processing and Presentation of Peptides Associated with MHC Class II**

Antigenic presentation associated with MHC class II proteins is carried out by APCs (Fig. 2B). These capture antigens from outside the cell by endocytosis and they present them to CD4<sup>+</sup> T-lymphocytes.<sup>19,20</sup> The interaction between CD4<sup>+</sup> T-lymphocytes and APCs takes place in the peripheral lymphoid organs, where specific cells for peptide/MHC class II complexes will become activated. These cells may act as effector cells in cell-mediated immunity processes and/or provide stimuli that are necessary for the proliferation and differentiation of B lymphocytes and cytotoxic T cells. After the internalisation by APC endocytosis, antigenic proteins are transported to the endosomes, vesicles with very low pH containing proteolytic enzymes. Intracellular trafficking of these vesicles carries them to the lysosomes, helping to accelerate the process of proteolysis. MHC class II  $\alpha$  and  $\beta$  chains are synthesised in a co-ordinated way and are assembled in the ER. These recently formed dimers are unstable and ER chaperones, such as calnexin, contribute to their processing and assembly. The invariant chain (Ii) is also bound to the ER heterodimers, occupying the peptide binding site and interfering with this binding. This blockage guarantees that class II molecules are not able to be loaded and present peptides that are in the ER. The Ii also drives class II molecules towards specialized endosomes, where the proteins that have been internalised have been proteolytically degraded into peptides. En route to the surface of the cell, exocytic vesicles transport class II molecules out of the ER and fuse with endocytic vesicles containing internalised and processed antigens, bringing these types of molecules into contact. The Ii must be eliminated in order that peptide/MHC class II complexes can form. This degradation is carried out by the same proteolytic enzymes as act on the internalised proteins, with only a fragment of 24 amino acids, known as class II-associated invariant chain peptide (CLIP), being left intact. The protein HLA-DM acts as a peptide exchanger, facilitating the removal of CLIP and the addition of other peptides to the MHC class II molecules. HLA-DM, encoded by genes in the MHC region, has a similar structure to the class II molecules and is found in the

same cell compartments. On the other hand, it is not a polymorphic molecule, nor is it associated with Ii or expressed on the surface of the cell. A molecule similar to HLA-DM, HLA-DO, is expressed in B-lymphocytes.

Class II molecules are stabilised by the binding to peptides and are exhibited on the surface of APC. These complexes have a long mean lifetime, increasing the probability of recognition by specific CD4<sup>+</sup> T cells and the interaction of the CD4 coreceptor with the nonpolymorphic regions of class II molecules. On the other hand, it has been shown that a very small number of peptide/MHC class II complexes is necessary to achieve the CD4<sup>+</sup> T-lymphocytes activation.

Although the majority of peptides bound to class II molecules come from internalised proteins from the extracellular environment, on occasions some cytoplasmic and membrane proteins may enter into the MHC class II pathway. These proteins are trapped in vesicles, known as autophagosomes, which come from the ER. They fuse with lysosomes where they are proteolitically degraded. In this way, even proteins belonging to pathogens that replicate in the cytoplasm of infected cells may be presented by class II molecules. In addition, the presentation of extracellular antigens by class I molecules has also been demonstrated. Accordingly, peptides recognised during the alloresponse can derive from both pathways, and they are presented by class I and class II molecules.

## BINDING OF PEPTIDES TO MHC MOLECULES

The binding of peptides to MHC molecules is not very specific. Every molecule, whether class I or class II, has a peptide-binding groove in which various peptides can fit.<sup>21</sup> This fact is not surprising, since every individual has a limited number of HLA molecules and these must be capable of presenting peptides from great number of antigenic proteins which they may encounter.

One of the differences between the peptides that are presented by MHC class I and class II molecules is their length. Class I molecules are able to present peptides between 8 and 11 amino acids, while class II are able to bind peptides between 10 and 30 residues, although the optimal length is between 12 and 16 amino acids. Also, the peptides that are binded by certain allotypes must present certain residues that allow complementary interaction between the peptide and the molecule; these amino acids are not the same as those recognised by TCR.

The association of antigenic peptides with MHC molecules is a saturable, low affinity interaction with slow liberation rate. This latter fact enables peptide/MHC complexes to remain on the surface of the presenting cells for a long time, ensuring their interaction with specific T cells. The peptides bind MHC molecules in extended conformation, certain peptide residues bind to pockets in the groove by hydrophobic interactions with complementary amino acids. These peptide residues, known as anchoring residues, are generally located in the middle or at the end of the peptide. Each MHC binding peptide often contains only one or two residues of this type; accordingly the rest of the molecule, including the zone recognised by the T-lymphocyte, can present a great variability. Not all the peptides use anchor residues for binding to MHC molecules, especially in the case of class II. Specific interactions of the peptide with  $\alpha$ -helical regions of the groove also contribute to the binding by hydrogen bonds and saline bridges. The peptides that bind to the MHC class I molecules generally have hydrophobic or basic amino acids in their carboxyl terminal end that contribute to the interaction. Additionally, there are restrictions

on the amino acids which interact most strongly, specifically those at positions 2 and 9 of the peptide, these being the anchors for the class I molecule.

The residues that are close to the MHC molecule groove are polymorphic among different allotypes, and consequently different alleles favour the binding of different peptides. One portion of the peptide is exhibited to the outside of the MHC molecule and the sequence of amino acids will be recognised by specific TCRs. TCRs also interact with the polymorphic residues of the MHC molecule. Thus, both the antigenic peptide and the MHC molecule contribute to the recognition of the T cell.

MHC molecules present both foreign peptides and self-proteins. However, the characteristics of the biosynthesis and assembly of the MHC molecules and the high specificity and sensitivity of T cells to peptide/MHC complexes enable foreign peptides to be recognised and autoimmune responses avoided.

## INTERACTIONS BETWEEN TCR-MHC

The receptor for the antigen present in the majority of T cells is a heterodimer composed of two transmembrane polypeptide chains, called  $\alpha$  and  $\beta$ , covalently bound by two disulphur bridges.<sup>22</sup> Each of them presents an N-terminal immunoglobulin variable domain (V), a constant immunoglobulin domain (C), a transmembrane hydrophobic region, and a short cytoplasmic tail. Variable regions in both chains have short amino acid sequences, where the variability among the various TCR residues, known as Complementarity Determining Regions (CDRs). Three juxtaposed CDRs in each chain form the structure that specifically recognises the peptide/MHC complexes. The domain V $\beta$  has a fourth hypervariable region that seems not to participate in the antigen recognition, but is a binding site for certain microbial products known as superantigens.

The interactions between TCR of the recipient and the MHC of the donor during allorecognition are similar to those occurring during conventional foreign peptide/self-MHC molecule recognition.<sup>23,24</sup> The structural similarity between the various peptide/MHC complexes and the TCR conformational flexibility can explain the occurrence of **degenerate interactions** between the two molecules.<sup>25,26</sup>

The flexibility of the TCR has been demonstrated in experiments in which re-organisation of the peptide binding region in the CDR3 has been observed, when the same peptide was presented by different MHC molecules. This CDR3 region seems to play a crucial role in the re-organisation of the whole TCR molecule.

## ALLORECOGNITION THEORIES

According to what we have seen above, T cells recognise the antigens in the form of peptides bound to MHC molecules, which may display both foreign and self-peptides. During the thymic selection process, T cells initially recognise peptides bound to MHC molecules that are expressed by antigen-presenting cells (APCs). Immature T cells with antigen receptors (TCR) that are capable of binding with “sufficient” affinity to self-peptide-self-MHC complexes receive a survival signal known as positive selection. These cells represent the MHC-restricted T-cell repertoire. On the other hand, by the negative selection process, potentially autoimmune T cells, with high affinity and/or with cross reactions with the aforementioned complexes are destroyed. Mature T cells

therefore have the ability to trigger immune responses when they recognise foreign proteins bound to MHC own molecules. Autoimmunity to self-antigens is avoided by a mechanism of self-tolerance.

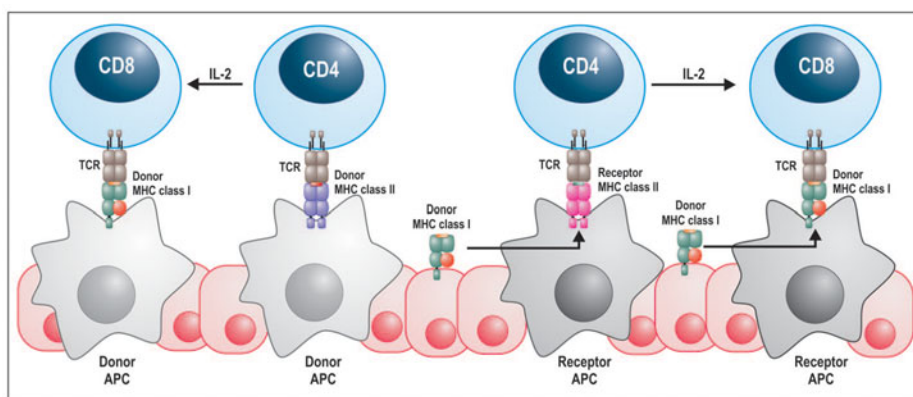
The special case of foreign MHC antigen recognition is known as allorecognition and consists of the capacity of T cells to recognise peptide/MHC complexes with which they have not been in contact during the process of maturation in the thymus.<sup>27</sup> A notable characteristic of the allorecognition process is the high number of alloreactive T cells (1 in  $10^3$ - $10^4$ ), while the number of T cells that are specific for single self-MHC/foreign peptides is between hundred and thousand order of magnitudes lower.<sup>28</sup>

There are several mechanisms of allorecognition.<sup>29-31</sup> In direct allorecognition, T cells recognise antigenic determinants in the MHC molecules that display on the surface the transplanted cells. In indirect allorecognition, MHC molecules of the donor are processed and presented as peptides by the MHC molecules of the recipient, in a similar way to conventional antigen processing. A third mechanism, known as semi-direct allorecognition, has been described recently: the dendritic cells of the recipient acquire complete peptide/MHC complexes transferred from the donor's cells.

### Direct Allorecognition

For a long time this was thought to be the only mechanism by which allogeneic antigens were recognised and, while it is now known that there are others, it is still considered the most common one. Two theories have been proposed to explain the molecular mechanisms that are responsible for this predominance. These differ with respect to the importance given to the presence of the peptide in the foreign peptide/MHC complex.

The “high determinant density” model proposes that alloreactive T cells are capable of **directly recognising polymorphic residues**, displayed in MHC molecules, attributing a lesser degree of importance to the peptide that is bound to the molecule<sup>32</sup> (Fig. 3). According to this, if every MHC molecule can serve as a ligand for one allospecific T cell, the density of antigens present on the surface of the cell is much higher than



**Figure 3.** Allorecognition pathways. In the direct pathway, the donor MHC molecule or the peptide that presents, it is recognized by T cells of the receptor. In the indirect route peptides from donor MHC proteins are presented to receptor T cells by their own MHC molecules in the surface of antigen-presenting cells.

what could be found for a specific peptide. The high density of ligands available to stimulate alloreactive T cells implies that low affinity receptors may be responding to foreign MHC and leading to a high frequency of alloreactivity. The blockage of the regions that contact the TCR in the MHC molecule with synthetic peptides or site-specific mutations may inhibit specific alloresponses. Further, alloreactivity has been detected against MHC molecules in the absence of peptides or independently of the peptides presented.<sup>33,34</sup>

The “multiple binary complex” model suggests that the **peptide recognition** bound to the allogeneic MHC drives direct allorecognition<sup>35</sup> (Fig. 3). Many different peptides together, in combination with an MHC allogeneic molecule, may produce determinants that are recognised by different T cells with cross-reactivity. Despite the peptides deriving from seric or cellular proteins and being processed naturally, they are different to those binding to the recipient MHC molecules due to polymorphic variations in the binding groove of the peptide. If each bound peptide is an essential component of the determinant recognised by alloreactive T cells, each allogeneic peptide/MHC complex is recognised by a different alloreactive T cell, and a single MHC incompatibility would trigger a great variety of T cells.<sup>36</sup> Alloreactive T CD8<sup>+</sup> cells that are specific for self-peptides presented by allogeneic class I molecules have been found, while there seems to be no evidence of peptide independent components. Moreover, the displacement of endogenous peptides from allogeneic APCs by incubation of exogenous peptides leads to loss of allorecognition by the allospecific T cells.

Alloreactivity, therefore, can be explained by the interactions occurring between TCR and allogeneic molecules that present polymorphisms or by interactions with the peptide presented. However, both models predict the existence of TCR/peptide/MHC structures that have not been observed by conventional antigenic recognition, implying that there are specific interactions with the peptide and with the self-MHC molecule.

It is possible that direct allorecognition may occur by both mechanisms, with a variable contribution from each depending on the site and degree of similarity of MHC molecules between stimulating and responding cells. In the case of great structural differences, the alloresponse may be directed towards MHC residues (high determinant density). If the donor and recipient are closely matched, the epitopes of endogenous peptides (multiple binary complexes) will be the focus of alloreactivity.

### Indirect Allorecognition

There is a great deal of evidence concerning the involvement of this type of allorecognition in the rejection of transplants.<sup>37,38</sup> Studies on heart, kidney and liver recipients have shown a strong correlation between in vitro detection of indirect responses and clinical rejection of organs. Cell and membrane fragments, as well as dead cells coming from the transplanted organ, will be phagocytosed by the APC of the recipient and the peptides will be processed and presented by self-MHC molecules<sup>39,40</sup> (Fig. 3). Exogenous antigens are mainly presented by MHC class II molecules and, therefore, the response to indirect presentation will be dominated by T CD4<sup>+</sup> lymphocytes. The frequency of T cells involved in indirect recognition is two orders of magnitude lower than those for direct recognition. However, the generation of multiple epitopes by alloantigen processing may be responsible for a substantial amplification of the response. On the other hand, it is a slower process than direct allorecognition and becomes important once the direct responses have been “exhausted”.<sup>41</sup>

## ALLORESPONSE AND IMMUNOLOGICAL REJECTION

The two types of allorecognition can lead to rejection of the transplant. Direct recognition prevails during the first few weeks or months after transplantation, and is caused by the APCs of the donor. These cells start disappearing from the transplanted organ and indirect recognition becomes important. There is evidence that the indirect pathway is sufficient to mediate both acute and chronic rejection.

When an alloantigen is recognised by any of these pathways, it triggers an effector response by the immune system known as the **alloresponse**. Both innate and adaptive systems work together in the process of rejection through a series of non-exclusive responses.<sup>42</sup> CD4<sup>+</sup> or CD8<sup>+</sup> T-lymphocyte act as cytotoxic cells against cells presenting the donor's MHC molecules, following the direct recognition pathway. Additionally, T-lymphocytes will stimulate delayed hypersensitivity mediated by macrophages, following activation by both direct and indirect pathways. In general, the production of cytokines Th1 and Th2, the activation of NK cells, the production of alloantibodies and the activation of the complement system are also mechanisms which tend to lead to destruction of transplanted tissue. Classically, it has been accepted that alloantibodies were involved in hyperacute rejection, while other cellular mechanisms mediated chronic rejection. However, recently, the humoral theory, supported by Terasaki, has gained support and it is currently accepted that humoral mechanisms are also involved in long-term rejection.<sup>43</sup>

### Hyperacute Rejection

This results in early loss of the transplanted organ, within the first 48 hours after transplantation. It occurs when there are preformed antibodies in the serum of the recipient against antigens expressed by the vascular endothelium of the transplanted organ. The antibodies involved are IgM class, specific for the antigens of the blood groups, or IgG, targeted to MHC antigens. In renal transplants, the occurrence of neutrophil infiltration in the glomerular and peritubular capillaries, destruction of the renal endothelium, interstitial haemorrhage and renal vasculature thrombosis, followed by cortical necrosis, have been observed. Hyperacute rejection has been also described in heart and lung transplants, and even in liver transplants, although this latter-type of transplant seems to be less vulnerable to this process.

The existence of anti-MHC antibodies involves the indirect pathway of activation of T cells. In general, alloantibodies are the consequence of previous immunizations, from blood transfusions, pregnancies, or previously rejected transplants. To avoid this type of rejection it is essential to carry out a full immunological screening, including determination of blood group, MHC typing, presence of alloantibodies and cross-matching between the donor and the recipient prior to transplantation.

### Acute Rejection

This type of rejection occurs between five days and three months after transplantation. In general histological findings show a diffuse interstitial cell infiltrate composed of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, with activated or memory phenotype. In other cases of acute rejection, such in vascular rejection, infiltrating cells are mainly macrophages. Recent research, initially on renal and cardiac transplants, has demonstrated that antibody-mediated



mechanisms also contribute to a great number of acute rejections. The antibodies involved seem to be IgM class, both for MHC class I and class II. Alloantibodies may be also specific against minor MHC antigens. The development of staining techniques to detect deposits of complement activation proteins C3d and C4d, in biopsies of the transplanted organs, has enabled this type of rejection to be studied.

### **Chronic Rejection**

Initially this term was used to describe the slow deterioration of the function of the transplanted organ. However, more recently it has come to be used only to refer to the late loss of the transplant as a consequence of the recipient's immune response against the organ.

In general, it is accepted that B cells are involved in this type of rejection, since deposits of C4d in presence of donor-specific alloantibodies have been detected. In fact, the development of alloantibodies in receptors of cardiac, renal and pulmonary transplants is related to the development of chronic rejection. The main damage to the organ, principally fibrosis, is the result of a complex process in which pathogenic effects add to the repair response, alloresponse and immunodepression.

Whatever the effector mechanism involved in the loss of the transplant, what has been demonstrated is that there are immunological factors in the recipient that are going to determine this process. Specifically, it has been observed in the case of renal transplants, the existence of clear differences in the maintenance of the organ five years after transplantation depending of the number of different alleles of the HLA-A, HLA-B and HLA-DR loci in the donor and the recipient. Also, the presence of preformed alloantibodies in the serum of the recipient is a clear risk factor for rejection, as we have pointed out earlier, and immunosuppressant treatment for these patients is carefully detailed in protocols. Basically, it is very important to monitor the state of the immune response to the transplant in these patients. Apart from the aforementioned technique for staining the C4d complement, other techniques are being evaluated, including the measurements of cytokines using ELISPOT assays or intracytoplasmatic staining, of ATP levels using the Cylex® method, and of lymphocyte activation markers using flow cytometry.

### **CONCLUSION**

The high degree of polymorphism of the MHC and the large number of genes involved, increased by the codominant expression of the proteins produced, generate a large variability that presents a considerable barrier to tissue and organ transplantation. Allorecognition by the recipient leads to a powerful anti-donor immune response, eventually leading to the rejection of the transplanted organ. The alloreactivity observed may be explained by the interactions between TCR and allogeneic MHC molecules presenting polymorphisms, by interactions with peptides presented by these molecules, or by the indirect presentation of peptides from allogeneic MHC molecules. Moreover, peptide recognition may be highly specific or highly degenerate, which may explain why allorecognition processes are so common. In any case, whatever the mechanism by which rejection is triggered, the severity and type of alloresponse is going to depend on the number and magnitude of differences in MHC molecules between the donor and the recipient. In addition, the existence of preformed specific alloantibodies against

the donor's MHC, or the development of thereof, will determine the evolution of the transplant. Despite immunosuppressive treatments being used with relative success, we still have still a long way to go to minimize the morbidity and mortality associated with transplantation. In relation to this, a better understanding of the processes of allorecognition and alloresponse, which will contribute to the development of new treatments, as well as the induction of tolerance mechanisms, will be essential to achieve long-term success of transplanted organs.

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## CHAPTER 4

# CELLULAR IMMUNOTOLERANCE IN THE TRANSPLANT

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**Abstract:** In humans, a state of operational tolerance has been observed in some recipients who anecdotally or experimentally abandoned their immunosuppressive treatment. Besides, advances in the understanding of the immune response and the continuous appearance of new biological molecules have boosted the growing interest in transferring the knowledge concerning immune tolerance from experimental models to clinical transplantation. Most of the strategies for inducing tolerance target the T-lymphocytes, especially T CD4<sup>+</sup> since they play a central role in the regulation of the immune response. However, an effective tolerogenic treatment must also take into account the role of alloantibody producing B-lymphocytes, which have been shown to play a fundamental role in chronic rejection phenomena. There are multiple regulation and silencing mechanisms that operate both during lymphocyte ontogeny in the bone marrow and thymus (central tolerance) and in the periphery (peripheral tolerance). These regulatory mechanisms include the destruction of APCs by cytotoxic lymphocytes, suppressive cytokines, and activation-induced cell death, among others. However, the mechanism that in recent years has come to be attributed the greatest role has been the active suppression of the response by T-lymphocytes themselves. These lymphocytes are named as regulatory T cells that include Tregs CD4<sup>+</sup>CD25<sup>+</sup>, Tr1 cells and Th3. The great therapeutic potential of regulatory lymphocyte populations for the control of allogeneic rejection is evident and several clinical trials in humans have been started to be implemented using populations of both Tregs and Tr1 cells for the prevention of allogeneic reactions.

## INTRODUCTION

The main objective of organ transplantation is to achieve long-term acceptance of the allograft with minimal or indeed without pharmacological immunosuppression. This is what is known as tolerance to the allograft or allotolerance. Ideally, this allotolerance should exist in the context of an immune system that has properly functioning antitumor and allergic responses and reactions to microorganisms, etc. In human transplantation, a state of tolerance has been observed in some recipients who anecdotally or experimentally abandoned their immunosuppressive treatment, mainly in the case of liver transplants. In recent years, there has been great progress in our understanding of the multiple mechanisms involved in the achievement of allotolerance, and this has led to the development of strategies for inducing tolerance.<sup>1</sup> While these strategies have been found to be successful in murine experimental models, they have seemed less effective in clinical trials with humans. So far, most therapeutic approaches target T-lymphocytes. However, an effective tolerogenic treatment must also take into account the role of B-lymphocytes in alloantibody production as well as components of the innate immune system. It should be noted that the divisions between innate and acquired, as well as cellular and humoral, responses are artificial and merely serve to help us understand the system, while in an individual they are all perfectly connected and regulated. In this chapter the most important evidence to date in this field will be reviewed.

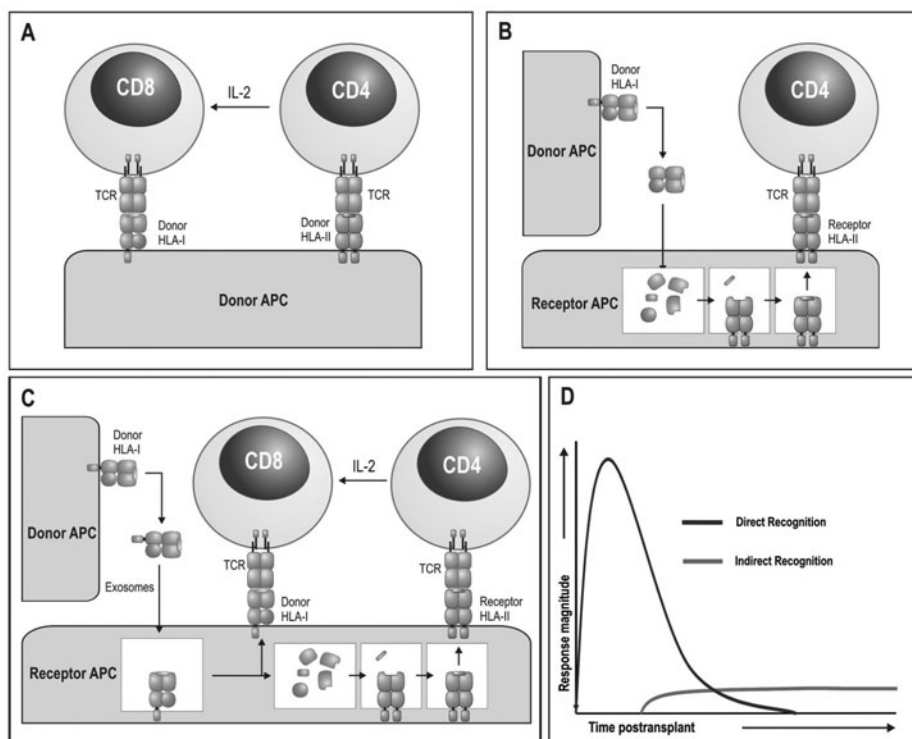
## EFFECTOR ALLOIMMUNE RESPONSE

### Allorecognition

In order to intervene in the mechanisms involved in the development of immunological allotolerance, it is necessary first to understand the basis of the immune response that is responsible for allogeneic rejection.<sup>2</sup> In general, this rejection occurs as a consequence of the uncontrolled activation of the immune system.

The vascular endothelium of the allograft is the boundary at which the immunocompetent cells of the host first come into contact with those of the donor. One of the earliest assaults which occur during the event of graft rejection is endothelial activation. This is accompanied by the expression of many surface molecules and the secretion of cytokines and chemokines which go on to directly regulate the inflammatory process. Essentially, the vascular endothelium of vascularised allografts is able to influence the rejection mediated by alloreactive T-lymphocytes of the host through two main mechanisms. Firstly, the endothelium plays a central role in the regulation of the extravasation of effector T-lymphocytes to the parenchyma of the graft. Secondly, endothelial cells participate directly in the activation of alloreactive T-lymphocytes by presenting alloantigens and supplying costimulation signals.<sup>3</sup>

The role of the innate immune system in allogeneic rejection has been clearly demonstrated in recent years. The transplant in itself means an injury that induces the activation of several innate immune response signals. In this way, according to the danger theory proposed by Polly Matzinger, soluble mediators of the innate response perpetuate the inflammatory state and promote the development of the acquired response. The activation of the complement cascade, apart from its involvement in the pathogeny of humoral lesions, is able to modulate the alloreactive T-cell response, probably by acting on the dendritic cells. In addition, Toll-like receptors (TLR) have recently been shown to be



**Figure 1.** Direct, indirect (A) and semi-direct (B) pathways of allorecognition. The semi-direct pathway is important in that it is capable of stimulating CD4<sup>+</sup> T cells (through the indirect pathway after processing the donor MHC molecules and presentation by the recipient MHC molecules) and CD8<sup>+</sup> T cells (through direct recognition of the vesicles directly transferred from the donor's APC to the recipient's). In this sense, there is a collaboration between the two types of response in semi-direct allorecognition. The contribution of this type of allorecognition to the allograft rejection is not well understood, but it is thought that direct allorecognition, that can be induced rapidly, is responsible of the early response, while indirect allorecognition, which needs antigen processing and therefore relatively more time, is involved in late response.

involved in the activation of immune response cells during acute allogeneic rejection.<sup>4</sup> Lastly, the role of NK cells in allogeneic rejection is still controversial. There is evidence of involvement of NK cell populations in the induction of tolerance to alloantigens after blocking of the costimulation, while, on the other hand, NK cells represent a significant barrier during the transplant of hematopoietic cells.<sup>5</sup>

With regards to the specific immune response, there are two types of donor alloantigen recognition of recipient T-lymphocytes<sup>2,6</sup> (Fig. 1). The first type is the so-called direct recognition, in which CD4<sup>+</sup> and CD8<sup>+</sup> T-lymphocytes of the recipient recognise peptide determinants directly from the intact MHC molecules of the donor expressed on the surface of the graft cells. The frequency of this type of alloreactive T-lymphocytes that recognise alloantigens by the direct pathway is very high, and there is also a great diversity of antigen receptors. In parallel to this recognition, there is a second type called the indirect pathway.<sup>2,6</sup> In this indirect recognition, donor's MHC molecules are processed and presented as peptides by the antigen presenting cells (APCs) of the recipient. This type

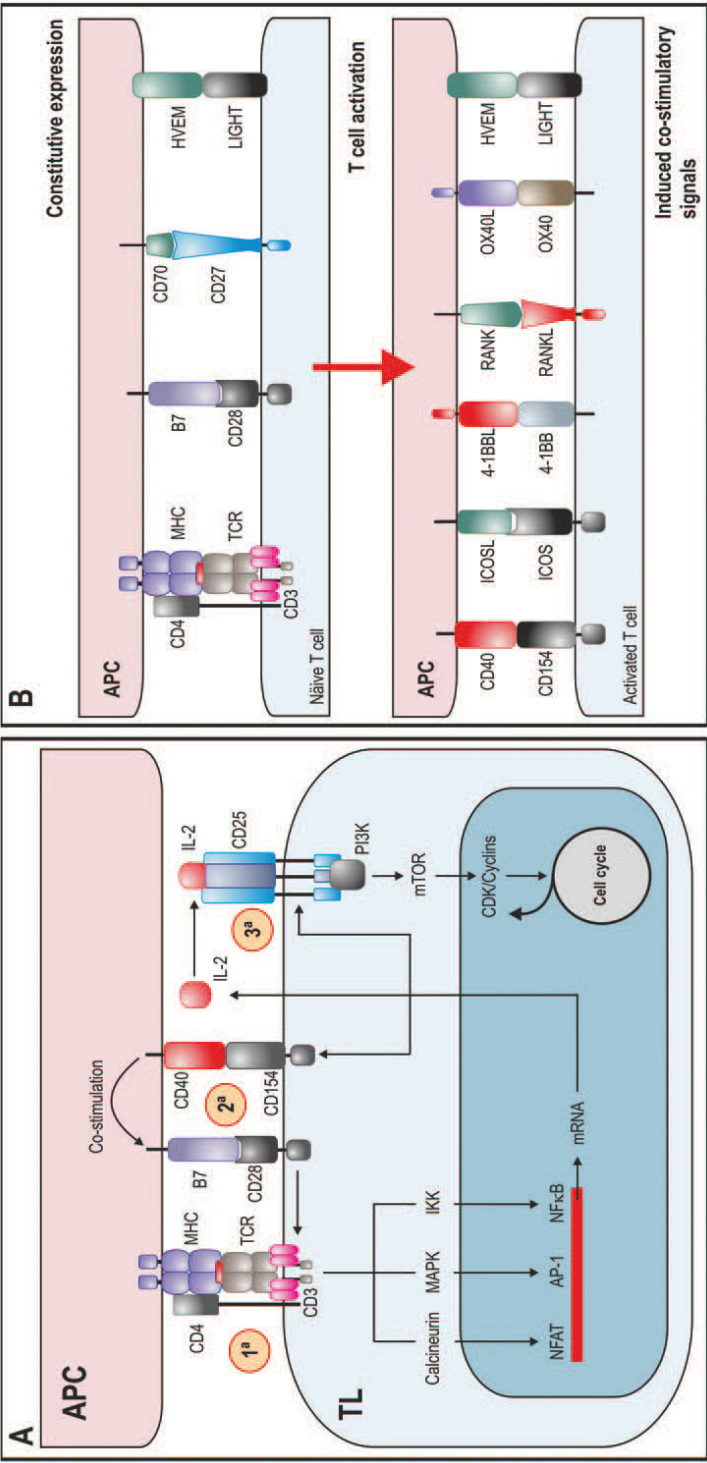
of immune response is therefore a self-MHC restricted response. In comparison to direct recognition, the frequency of alloreactive T cells of the indirect pathway is much lower (equivalent to any other cell with reactivity against a specific peptide) and the repertoire of T-cell receptors (TCRs) used is much more limited. The contribution of each of the recognition pathways to the regulation of the allogeneic response is largely unknown. Mixed lymphocyte culture that is commonly used to determine degree of alloreactivity reflects the intensity of direct alloresponse. In recent years evidence has emerged showing how intact MHC molecules may be transferred between cells of the immune system and stimulate a T-lymphocyte response in the recipient by semi-direct allorecognition<sup>6</sup> (Fig. 1). This type of response means that we can add to the list a so-called four-cell model in which CD8<sup>+</sup> T-lymphocytes are only stimulated through direct recognition by APCs of the donor while CD4<sup>+</sup> effectors and regulators are recruited through the indirect pathway by the APCs of the recipient. In this semi-direct recognition, recipient APCs capture intact MHC-peptides and stimulate CD8<sup>+</sup> T-lymphocytes through direct recognition, while these same APCs are able to induce indirect recognition by CD4<sup>+</sup> T-lymphocytes. Direct recognition is fast, and therefore it prevails in the alloimmune response in the period post-transplant, while indirect recognition, which needs more time to act, dominates in the longer term<sup>6</sup> (Fig. 1).

### Costimulatory Signals

In addition to the interaction between the host's T-lymphocytes and the alloantigen, the presence of other accessory signals is necessary for the effective activation of alloreactive lymphocytes. These costimulatory signals, known in general terms as the "second signal", are mediated by the interaction between nonpolymorphic receptors expressed on the surface of alloreactive T-lymphocytes and their respective ligands on the surface of the donor (direct pathway) or the recipient APCs (indirect and semi-direct pathways).<sup>6,7</sup> The absence of these costimulatory signals may lead to toleration rather than the activation of alloreactive T-lymphocytes. Among the possible cellular interactions between the two cell populations, it is particularly worth mentioning the interaction between the CD28 family (CD28, CTLA-4, ICOS, OX40) and ligands of the B7 family (B7.1, B7.2, B7-H1, ICOSL, OX40L), and between CD40L (CD154) and CD40<sup>8</sup> (Fig. 2). All these molecules, an ever expanding list, may be constitutively expressed or induced both in T-lymphocytes and in activated APCs. At the same time, costimulatory signals are regulated by inhibitory signals induced by molecules such as CTLA-4 (CD152) or PD1, when they bind to their ligands (B7-1 and B7-2 or PDL-1 and PDL-2, respectively),<sup>8</sup> allowing the response to decrease after initial activation of the T-lymphocytes. If the activation is partial, T-lymphocytes die via apoptosis, or become anergic, a state in which they do not respond to proliferation signals.

The induction of the first and second signal activates three intracellular signal transduction pathways: Calcium/calcineurin, Ras/MAP kinase and NF- $\kappa$ B. These signals induce the expression of various molecules, key among which are IL-2 and its receptor (including CD25, the alpha chain of this receptor). The IL-2 signalling of the receptor gamma chain, through JAK3, activates PI3-kinase, which in turn activates mTOR. This constitutes the third signal and triggers the cell cycle initiating the proliferation of alloreactive T-lymphocytes, which need nucleotides for the synthesis of DNA.<sup>9</sup>

The molecular basis of the progressive deterioration of the graft have been studied recently with using microarrays to systematically study gene expression in biopsies obtained from kidney transplants and a variety of different patterns have been observed. These differences reflect the kind of cell that infiltrate in the rejected graft and their degree of activation, and



**Figure 2.** The activation of T-lymphocytes in rejection of allografts requires three types of signals (A). The first signal is induced after the specific recognition of the alloantigen. The complete activation of the T cell requires costimulatory signals (second signal) that induces intracellular transduction pathways, responsible for the synthesis of IL-2, among others. This cytokine, through autocrine signalling, binds to its receptor on the surface of the lymphocyte (CD25 being the alpha chain of this receptor) and induces the start of the cell cycle and the proliferation of lymphocytes. In the case of the activation of lymphocytes in transplantation, there is a series of molecules that induce more specifically, belonging to the family of B7 and TNF (B), which are the targets of the new treatments being developed to avoid transplant rejection.



show that in a large number of cases, antibody producing B-lymphocytes determine the patterns of expression prevailing in the biopsies, demonstrating the importance of controlling the humoral response in the evolution of allografts.<sup>10</sup> These studies have revealed, according to the predominant pattern of expression, the existence of at least three different types of acute rejection. Specifically, one group of patients has higher functional activity of genes related to the accumulation and activation of T-lymphocytes and apoptosis, while others are found to have a pattern of expression dominated by the genes involved in innate immunity, and in a third group expression of genes involved in the cellular cycle prevails.<sup>10</sup> All this seems to indicate the great complexity of the mechanisms that lead to rejection, and at the same time, the primacy of immunobiological factors.

For a long time, it has been known that the presence of specific antibodies against the donor before the transplant in the recipient serum targeting molecules of the human major histocompatibility complex (HLA) determine the appearance of a hyperacute rejection. This is why it is necessary to perform a cross-matching test prior to the transplantation of renal transplants, and check whether there are preformed antibodies, in the case of heart transplants. However, the post transplant production of these antibodies and other antibodies against alloantigens of the donor may play a crucial role in evolution of the graft. In fact, humoral rejection has been implicated as a main cause of long-term graft loss of the kidney (graft chronic nephropathy), lung (bronchiolitis obliterans) and heart (allograft coronary vasculopathy).<sup>11</sup>

### **Chemokines and Cytokines**

Chemokines, a family of at least 48 low molecular weight molecules, are largely responsible for the leukocyte migration to tissues and to secondary lymphoid organs to trigger the alloimmune response explained above. Chemokines CCL9 and CCL21 seem to play a major role in the regulation of the migration of dendritic cells, the main APCs involved in the alloimmune response. Consequently, receptors that are deficient in CCL19 and CCL21 tolerate transplanted allogeneic islets in the renal capsule indefinitely. On the other hand, it has recently been demonstrated that the migration of alloreactive T-lymphocytes to the allograft is dependent on CXCR3 and CCR5, using specific monoclonal antibodies and CCR5 deficient mice respectively.<sup>12</sup>

The differentiation of naive T-lymphocytes into effector T-lymphocytes typically involves production of a specific group of cytokines. In fact, much of the progress achieved in the understanding of the function and development of T-lymphocytes has been linked to progress in our understanding of the biology of cytokines. Furthermore, cytokine production patterns were the basis on which two types of classical CD4<sup>+</sup> T-lymphocytes, Th1 and Th2, were identified. Indeed, currently, many different types of T-lymphocytes have been distinguished, thanks to the discovery of new families of cytokines and of new cytokines in existing families, as well as the identification of pleiotropic activity as in the case of IFN $\gamma$ .<sup>13</sup> This cytokine is characteristic of the Th1 response, is associated with allograft rejection and is essential in the majority of experimental models of allogeneic transplant rejection.<sup>14</sup> However, there are contradictory results from experiments with IFN $\gamma$ -deficient mice in that they reject the kidney faster than nondeficient mice. The reason for this is not known, although it is possible that other cytokines may provide an explanation, such as those involved in the response described recently, Th17, IL-17 and IL-23.<sup>14</sup> Other cytokines such as IL-10, TGF $\beta$  and IL-12 also seem to be important in the control of the alloresponse since their inhibition promotes the development of either acute or chronic allograft rejection.

## STRATEGIES FOR INDUCING TOLERANCE IN TRANSPLANTS

We have to take into account that the mechanisms to induce and maintain immune tolerance have been developed exclusively to prevent the immune response, but not the alloreactive response. The latter differs from the former in several respects: The immune response against alloantigens is a legitimate reaction of the body against foreign antigens; it also involves a large number of precursors (many more than employed against alloantigens) and has high affinity. Given all this, strategies for inducing self-tolerance would not necessarily be effective for inducing tolerance towards antibodies. However, experimental models have demonstrated that mechanisms of the immune system to maintain tolerance against self-antigens may be used to induce tolerance towards alloantigens.<sup>15</sup>

Most of the strategies for inducing tolerance target the T-lymphocytes. However, an effective tolerogenic treatment must also take into account the role of alloantibody producing B-lymphocytes, which have been shown to play a fundamental role in chronic rejection phenomena. On the other hand, the existence of a small number of transplanted patients who do not receive immunosuppressive treatment (in general due to voluntary abandonment or severe side effects) and yet do not suffer from rejection against the allograft indicates that, in certain circumstances, it is possible to induce long-term immunological tolerance in humans.<sup>16</sup>

In order to avoid the aberrant activation of all the self-reactive T and B-lymphocytes that are constantly produced and the appearance of autoimmune symptoms, there are multiple regulation and silencing mechanisms, all coming under the term immune tolerance, that operate both during lymphocyte ontogeny in the bone marrow and thymus (central tolerance) and in the periphery (peripheral tolerance). In this chapter we will focus on those tolerogenic mechanisms that have shown to be useful in allogeneic transplant and that have mainly targeted CD4<sup>+</sup> T-lymphocytes, the lymphocytes which play a central role in the regulation of all allogeneic responses.

### Central Tolerance

The process known as negative selection takes place in the thymus, in the case of T-lymphocytes, and in the bone marrow, for B-lymphocytes and is carried out through induction of apoptosis. This process is mediated by recognition between the self-reactive receptor and the alloantigen modulated by its intensity. In terms of the avidity of interaction and the number of antigen receptors involved (for self-antigen-receptor interactions of similar avidity, abundant self-antigens will induce a more intense clonal elimination than rare self-antigens). The mechanism of clonal selection in the thymus may be exploited in transplantation through exposing the thymus of the recipient to donor alloantigens (see later), which will induce the negative selection or central deletion of alloreactive T-lymphocytes that would develop specific tolerance towards the antigens of the donor. In fact, this was the basis of the experiments on induction of tolerance and cutaneous allograft acceptance, through the infusion of the donor cells in newborn mice, carried out by Billingham, Brent and Medawar. Indeed, the tolerance of allografts by these newborn mice illustrates the robustness of the central tolerance.<sup>17</sup>

As well as negative selection, CD4<sup>+</sup>CD8<sup>+</sup> precursors go through a process of positive selection by which those cells not capable of recognising and interacting with self-MHC molecules expressed on the surface of the epithelial cells of the thymus cortex are deleted by apoptosis. Since both MHC class I and class II molecules are unstable in the absence of



antigenic peptides and the majority of the peptides presented in the thymus are self-antigens, positive selection takes place on the basis of self-reactivity. Given that, surviving T-lymphocytes go through a second negative selection process in the thymic medulla. There, the CD4<sup>+</sup>CD8<sup>+</sup> precursors interact with epithelial cells of the medulla and dendritic cells of haematopoietic origin. When the self-MHC peptide recognition by CD4<sup>+</sup>CD8<sup>+</sup> lymphocytes reaches a certain level, programmed cell death, apoptosis, is triggered. At the end of the selection process, only those CD4<sup>+</sup> or CD8<sup>+</sup> T-lymphocytes that recognise their own antigens (MHC-self antigen) with a weak affinity have been allowed to reach maturity.<sup>17</sup>

The importance of the mechanisms of negative selection in the induction of immune tolerance is the basis of various strategies for the prevention of allogeneic rejection. The first of these strategies consists of the intrathymic injection of alloantigens (in form of peptides or cells). Suitable experimental models have shown that this treatment induces the effective removal of alloreactive lymphocytes. However, the maintenance of long-term tolerance requires the persistence of the antigen in the thymus. The second type of therapeutic approach intended to induce the negative selection of alloreactive precursors, is based on inducing double chimerism following the combined organ and bone marrow transplant from donor to recipient.<sup>18</sup> The success of this type of protocol depends on the induction of a sufficient degree of hematopoietic chimerism, sustained over time, to enable donor hematopoietic cells involved in the negative selection to remain in the recipient's thymus indefinitely. These protocols have been based on the experience in small number of cases of individuals suffering from leukaemia and/or myeloma who have received therapeutic bone marrow transplants and, after some years, develop kidney failure forcing renal transplantation. These patients have high graft survival rates (up to seven years) in the absence of immunosuppression, even though it is not possible to detect the presence of chimerism. In vitro studies on samples from these individuals confirm a humoral response against the donor but no cytotoxic activity against renal tubular cells of the donor is detected.

Despite all this, the evidence to date comes from a very select group of patients and there are projects underway which aim to achieve this type of tolerance in the absence of haematological malignancy and even the case of HLA incompatibility.

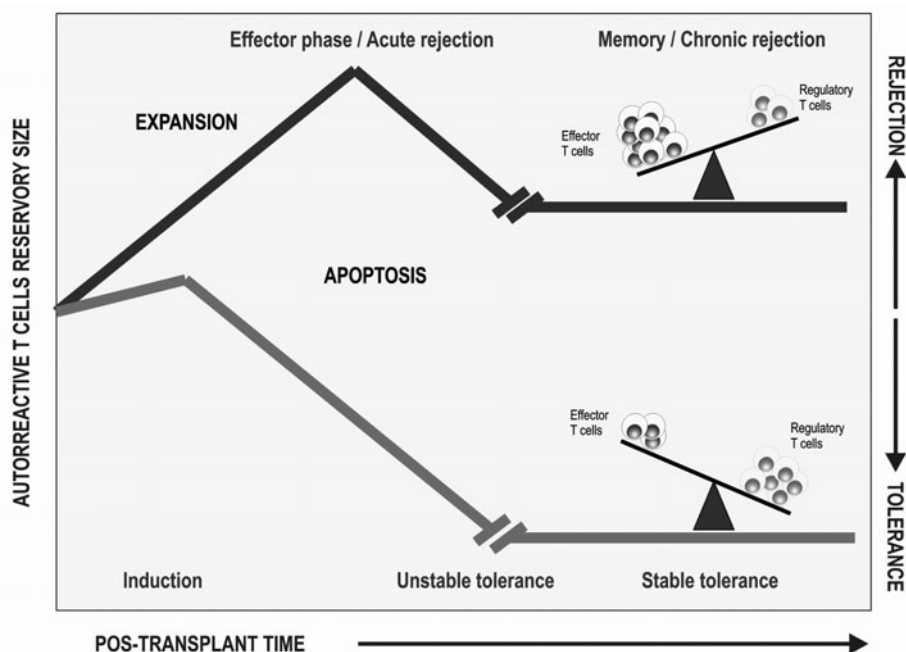
### **Peripheral Tolerance**

Central tolerance mechanisms are imprecise, since a very diverse repertoire of lymphocytes to recognise any antigen must be allowed to mature, otherwise the individual will run the potentially severe risk of lacking defence against microorganisms. Also, in the case of transplantation, in order to promote a state of tolerance, it is essential to minimise the number of alloreactive effector T-lymphocytes in the blood while alloreactive regulatory T cells are boosted (Fig. 3).

There are various different strategies for inducing peripheral tolerance in transplant models (and in some cases of human transplants).<sup>1</sup> The various mechanisms for peripheral tolerance are outlined below, with emphasis on the approaches that have been tried in clinical and/or preclinical studies.

### **Anergy**

The concept of clonal anergy was described from experiments in which it was observed that the stimulation through TCR of CD4<sup>+</sup> T clones in the absence of a second signal induced a hypo-response (identified as the inhibition of the cellular proliferation



**Figure 3.** The balance between effector and regulatory T cells in transplantation. The number of regulatory T cells does not vary greatly during the post-transplant period. On the other hand, the presence of alloreactive effector T cells can increase as a consequence of allograft recognition. A large growth at the beginning of the transplant may lead to acute rejection. In the long-term, the evolution of the graft towards chronic rejection or tolerance will depend on the balance between the number of effector and regulatory T cells.

and the production of IL-2) against subsequent stimuli in the presence of costimulatory signals. The induction of anergy in T-lymphocytes occurs through several mechanisms: Lack of accessory or costimulatory signals, low-affinity antigen recognition, and antigen presentation by immature APCs.

Blocking these signals is a very appealing way to induce tolerance, since the agents used are antigen-dependent, and generally, have little or no toxicity. The first attempts at anergy induction in transplants were made in murine and primate experimental models: The blocking of the interaction CD40/CD40L, using monoclonal antibody (mAb) anti-CD154 seemed to be effective in terms of graft survival. However, these experimental results have not been successfully transferred to humans and clinical protocols have been abandoned due to a high prevalence of an adverse effect, thromboembolism, caused by the reactivity of mAb with activated platelets.<sup>7-9</sup>

On the other hand, the inhibition of the interaction of CD28 with its ligands in the APCs has been of greater clinical relevance. Specifically, belatacept (LEA29Y—the soluble form of the fusion protein CTLA4-Ig) increased graft survival times in experimental models and, in a Phase II clinical trial, was found to have better long-term results (lower incidence of chronic graft nephropathy and improved graft function after one year) than conventional treatments. Calcineurin inhibitors, such as ciclosporin, as well as producing poorer long-term results, inhibit anergy induction. Anergy is associated with an altered

intracellular signalling that is dependent on the activation and the nuclear translocation of transcription factor NFAT (Nuclear Factor of Activated T cells) regulated by calcineurin.<sup>7-9</sup>

The EGR (Early Growth Response) family of transcription factors is one of the transcription targets of NFAT. During anergy induction in T-lymphocytes, both in vivo and in vitro, the expression of EGR2 and EGR3 is seen to increase, and this reduces the transcription of IL-2 gene. The factors NFAT, EGR2 and EGR3 also regulate the transcription of three members of the E3 ubiquitin-ligase family: Cbl-b (Casitas B-lineage Lymphoma B), GRAIL (Gene Related to Anergy in Lymphocytes) and ITCH (Itchy homologue E3 ubiquitin protein ligase). There is solid evidence concerning the involvement of these three E3 ubiquitin-ligases in anergy induction in T-lymphocytes, through control of the CD28 signalling pathway, the organisation of the actin cytoskeleton, the establishment of the immunological synapse and the degradation of phospholipase C $\gamma$ 1.<sup>18</sup>

It should not be forgotten that anergy is reversible under inflammatory conditions, such as infections, and that therefore it should not be seen as a single mechanism of tolerance in transplants.

### **Inhibition of Memory T-Lymphocytes**

In looking to achieve tolerance to transplants, one of the cellular targets are memory T-lymphocytes. These cells have less strict activation requirements than naive T-lymphocytes, since they need fewer costimulatory signals, they have higher antigen avidity and they have greater capacity for migration.<sup>19</sup> That is why the induction of tolerance in animals with memory lymphocytes is difficult to achieve. On the other hand, even for the first transplant, naive lymphocytes are not the only cells responsible for the response against the allograft, since heterologous memory T-lymphocytes may have cross reactivity. Lymphoid irradiation combined with parental administration of thymoglobulin has been looked into as a method to achieve lymphocyte depletion. However, this procedure risks inducing homeostatic cell proliferation in memory T-lymphocytes and failing to achieve tolerance.<sup>19</sup> Moreover, regulatory T-lymphocytes are resistant to depletion. This was one of the explanations proposed for the controversial results obtained with Campath 1H (anti-CD52), that targets multiple cell types (among them memory T-lymphocytes) and complicates the development of tolerance.

### **Ignorance**

In some circumstances, antigens, despite being recognised by specific receptors seem to be ignored by T and B-lymphocytes. The first evidence of this mechanism as a mediator for tolerance arose from experiments on skin allograft acceptance in naive mice without secondary lymphoid organs. A proposed explanation is based on the inability of the donor antigens to activate T-lymphocytes outside secondary lymphoid organs. Another possibility is that antigens are presented by nonprofessional APCs that are unable to activate T-lymphocytes.<sup>6</sup>

Factors that determine clonal ignorance are, among others: The level of expression of the antigen, the time elapsed between leaving the thymic precursor and reaching the periphery, the existence of pro-inflammatory cytokines in the medium/environment and the presence of costimulatory signals in tissues. Clonal ignorance is a precarious state since any signal of danger or inflammatory stimulus may disturb it. Consequently, it seems reasonable to suppose that this mechanism may be of limited use in terms of inducing clinical tolerance against allografts.<sup>1</sup>

## Dendritic Cells

The important role of dendritic cell (DC) populations in the induction and maintenance of peripheral tolerance in the absence of danger signs (infections and pro-inflammatory stimuli) has recently been demonstrated.<sup>20</sup> These tolerogenic dendritic cells may be located within the T-cell areas of secondary lymphoid organs (especially DC CD8 $\alpha^+$ ) and in the marginal sinus or red pulp of the spleen (DC CD8 $\alpha^-$ ) from where they migrate to the interior of T-cell areas of the white pulp. Tolerogenic dendritic cells are at the semi-mature stage (they constitutively express low levels of MHC molecules and costimulatory molecules such as CD80 and CD86). In basal conditions, these cells survive for longer and are capable of processing and presenting self-antigens, that they capture with specific membrane receptors or after phagocytosis of apoptotic cells, without undergoing subsequent maturation processes (increase in the expression of costimulatory molecules and in the production of IL-12). They work by inducing apoptosis and/or clonal anergy in autoreactive T-lymphocytes or, as we will see later, by promoting the generation of regulatory T-lymphocytes.<sup>20</sup> On the basis of these findings, a great effort is being made to generate and propagate tolerogenic DC in vitro, using a battery of factors that block their maturation such as IL-10, TGF $\beta$  prostaglandin E<sub>2</sub>, neuropeptides, Vitamin D<sub>3</sub> and various immunosuppressive drugs. In some experimental protocols, the administration of DC produced in vitro in the presence of alloantigens, has been shown effective in preventing allogeneic rejection in murine models of cardiac transplantation.<sup>20</sup>

## Active Suppression and Regulatory T-Lymphocytes

Every element of the immune response must have its mechanisms of control to stop that response once the agent triggering it has been eliminated. The outcome of the any immune response then depends on the equilibrium between effector-activator and regulatory functions. There are several regulatory mechanisms including the destruction of APCs by cytotoxic lymphocytes, suppressive cytokines, and activation-induced cell death, among others. However the mechanism that in recent years has come to be attributed the greatest role has been the active suppression of the response by T-lymphocyte themselves.<sup>21</sup>

The existence of immune cells with suppressive capacity was proposed for the first time in 1970 by Gershon and Kondo. This initial idea was not only forgotten but even stigmatised in the nineteen-eighties and first half of the nineties on the basis of three important facts: The inability of the scientific community to identify specific populations with immunosuppressive activity; the unequivocal demonstration of the existence of negative selection mechanisms for self-reactive lymphocytes in the thymus and in bone marrow and anergy in the periphery; and the identification of functional subpopulations of CD4<sup>+</sup> T-lymphocytes producing different profiles of cytokines with capacity for mutual inhibition (CD4<sup>+</sup> T<sub>H1</sub> and T<sub>H2</sub>). However the existence of suppressive lymphocytes was eventually demonstrated in 1995 by Sakaguchi, who identified a small subpopulation of CD4<sup>+</sup> lymphocytes that constitutively expressed the CD25 molecule (the  $\alpha$  chain of the IL-2 receptor) and was capable of inhibiting the development of various autoimmune processes.<sup>22</sup> This discovery was made using an experimental model of autoimmunity, in the development of organ-specific pathologies such as thyroiditis, gastritis, oophoritis or diabetes after perinatal thymectomy (between the second and fourth day of life) in normal mice and/or after the infusion of lymphocytes CD4<sup>+</sup>CD25<sup>-</sup> (effector lymphocytes) in adult athymic mice (nude mice). Administration, at the time of the thymectomy or of

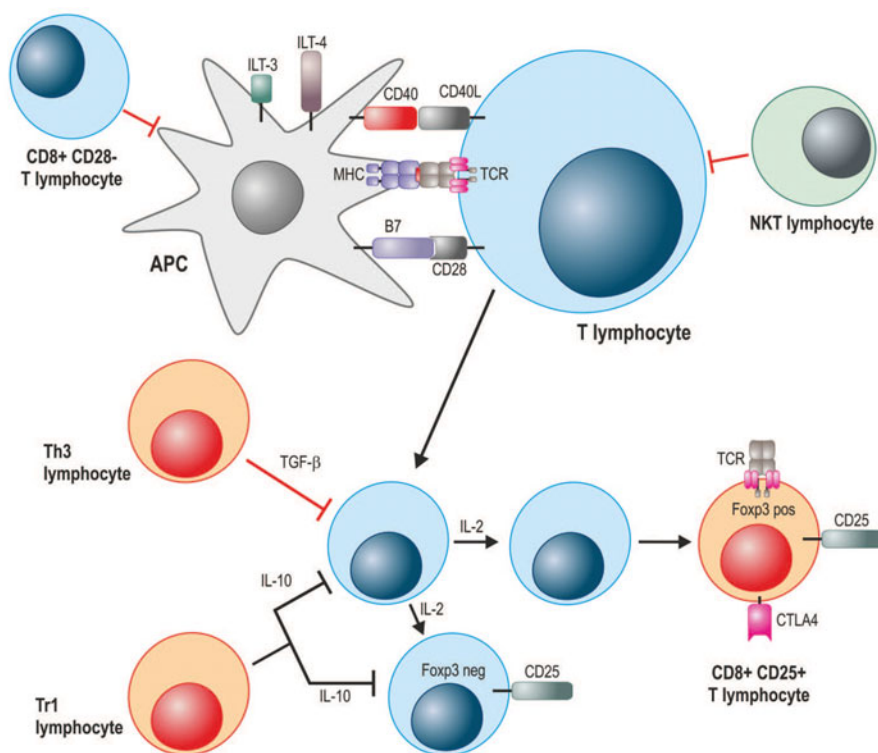
the injection of effector lymphocytes, of a small proportion of CD4<sup>+</sup>CD25<sup>+</sup> lymphocytes purified from the spleen or lymph node, prevented the development of these autoimmune diseases. The development of the disease after perinatal thymectomy suggested that CD4<sup>+</sup>CD25<sup>+</sup> lymphocytes had a thymic origin.<sup>22</sup>

#### *Regulatory CD4<sup>+</sup> T-Lymphocytes (Tregs)*

With respect to organ transplantation and its importance in the mechanisms of tolerance induction, the most interesting, of all the cell populations with regulatory/suppressive capacity (Fig. 4), is that of the regulatory T-lymphocytes which express CD25, also known as Tregs.<sup>23</sup> The expression of this molecule is very high in human Treg cells, for this reason they are denominated CD4<sup>+</sup>CD25<sup>high</sup>, in comparison to recently activated effector CD4<sup>+</sup> T-lymphocytes in which the expression is very low or temporary. Other markers have been described whose expression has served to define and select Treg lymphocyte populations such as CD45RB, CTLA-4, TNFRSF18 or GITR (glucocorticoid-induced TNF receptor family-related gene), CD134 (OX40) and CD62L. As happens with CD25, none of these molecules is exclusively expressed in Treg cells.<sup>23</sup> On the other hand, the expression of transcription factor FOXP3 in these lymphocytes is considered to be defining characteristic of these lymphocytes. Mutations in the FOXP3 gene cause a lethal autoimmune syndrome in *Scurfy* mice and are responsible for the IPEX (immunodysregulation polyendocrinopathy enteropathy X-linked) syndrome in humans. Both *Scurfy* mice and patients with IPEX have very low numbers of Tregs and impaired suppressive functions for these lymphocytes. The differentiation of the majority of peripheral regulatory T cells starts in the thymus after induction of FOXP3 gene expression in a subtype of TCRαβ with high affinity for self-antigen/MHC complex. These lymphocytes are known as natural regulatory T cells (nTreg cells), in contrast to Treg cells that can be induced (iTreg lymphocytes) under specific in vitro conditions, such as the antigen presentation by plasmacytoid dendritic cells in the presence of IL-2 and TGF-β. On the one hand, the transfection of FOXP3 into CD4<sup>+</sup>CD25<sup>-</sup> lymphocytes gives them suppressive capacity and, on the other hand, when FOXP3 is over-expressed in transgenic mice CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD8<sup>+</sup> lymphocytes become able to mediate suppression, both of which indicate that the expression of FOXP3 modulates the functional capacity of Treg cells. The importance of FOXP3 seems to be based on its ability to amplify and stabilize the transcription of specific regulatory genes, and thereby maintain homeostasis in Treg cells, rather than initiating the production of Treg cells. In mice the expression of FOXP3 is restricted to Treg cell populations, but not in humans, where this gene is expressed not only in recently activated naive lymphocytes, but also in memory lymphocytes. Recently it has been reported that the low expression of IL-7 receptor (CD127) can be used as a biomarker for Treg cells, which have the strongest suppressive effect and the highest expression of FOXP3.<sup>25</sup>

#### *Tr1 Cells*

In addition to Treg cells, there is another type of regulatory CD4<sup>+</sup> lymphocyte, the so-called Tr1 cells. These cells are produced in the periphery from naive CD4<sup>+</sup>CD25<sup>-</sup> lymphocytes in response to antigen stimulation under suboptimal costimulatory conditions. IL-10 and immature dendritic cells are among the best known inducing factors. Unlike CD4<sup>+</sup>CD25<sup>+</sup> Treg cells, Tr1 cells work through inhibitory cytokines such as IL-10 and



**Figure 4.** Schematic representation of the types of cells with regulatory capacity involved in the tolerance induction against the transplant.

TGF- $\beta$  and do not express FOXP3.<sup>24</sup> There are no characteristic markers on the surface of these cells, although they can be distinguished from Th2 cells by the pattern of cytokines released (IL-2<sup>low</sup>IL-4<sup>-</sup>IL-5<sup>+</sup>IL-10<sup>+</sup>TGF $\beta$ <sup>+</sup>). Also, while nTregs migrate to lymph nodes, Tr1 cells migrate towards sites of inflammation. Tr1 cells inhibit the response of naïve and memory T-lymphocytes, as well as the expression of costimulatory molecules and the secretion of pro-inflammatory cytokines by the APCs. The initial induction and activation of these lymphocytes is antigen-dependant but, once activated, their suppressive capacity is not antigen-specific.<sup>24</sup>

### Th3 Cells

A second type of induced CD4<sup>+</sup> T regulatory cells, known as Th3, are virtually identical to Tr1 in most respects, except that they secrete TGF- $\beta$ . Interestingly, it has been described how TGF- $\beta$ , a mediator of the effects of Th3 cells, is capable of transforming nonregulatory CD4<sup>+</sup>CD25<sup>-</sup> into regulatory CD4<sup>+</sup>CD25<sup>+</sup> T cells, inducing the expression of FOXP3. As is the case with Tr1 cells, there are no phenotypic markers that enable them to be identified.<sup>24</sup>

An important finding has been the discovery that the activation of CD4<sup>+</sup>CD25<sup>-</sup>FOXP3<sup>-</sup> T cells in the presence of TGF- $\beta$ , as well as inducing the production of iTreg cells, is able to stimulate the appearance of IL-17 producing CD4<sup>+</sup>CD25<sup>-</sup> T cells (T<sub>H17</sub> cells).<sup>13</sup> The



aberrant production and activation of these cells has been found to be involved with the pathogenesis of various immune and inflammatory disorders. Thus, TGF- $\beta$  may work either as an inducer of peripheral tolerance or as a pro-inflammatory factor. One explanation has been proposed for this ambivalent role of TGF- $\beta$  in the control of the functional differentiation of CD4<sup>+</sup> T cells. Namely, when cytokine IL-6 is present, a factor produced by many types of cells in response to pro-inflammatory stimuli, TGF- $\beta$  promotes the production of T<sub>H17</sub>.<sup>13</sup> However, in the absence of such pro-inflammatory stimuli, TGF- $\beta$  promotes the production of iTreg cells. It has also been noted that the presence of IL-2 boosts the capacity of TGF- $\beta$  to induce iTregs. Taken together, the aforementioned studies indicate that both the number and the functional activity of this population of suppressor T cells is regulated by a wide range of mechanisms, which undoubtedly reflects its importance in the homeostasis of the immune system.

#### *Other Cells with Regulatory Capacity*

In addition to those already described, many populations of immune cells have been reported to have regulatory activity in certain experimental models. These populations have been identified in the CD8<sup>+</sup> T lymphoid compartments and double negative T and B cells, as well as in NKs and DCs.<sup>24</sup> Most of these populations have not been studied extensively and their relative contribution to the overall level of suppressive activity remains unknown.

Mesenchymal stem cells (MSCs) have been attributed an important role as mechanism for inducing tolerance. These cells have pluripotent differentiation potential, are self-renewing and have the ability to produce new tissues and cells. Another advantage is that they can be obtained from tissue that is easy to access, such as bone marrow or umbilical cord.<sup>26</sup> From the immunological point of view, the great surge of interest in them is due to the apparent ability to suppress the immune response that has been demonstrated in vitro. MSCs are able to inhibit the proliferation of T cells against alloantigens and polyclonal stimuli, as well as to prevent the differentiation of cytotoxic T cells. On the other hand, the immunogenicity of these cells seems to be minimal, since at least in vitro they have been shown to induce virtually no proliferation of foreign lymphocytes.<sup>27</sup> This characteristic, together with their immunosuppressive properties, makes MSCs a tool with great potential to induce tolerance for organ transplantation. In fact, over the last three years, evidence has been mounting for their usefulness in experimental models of organ transplantation, including, for example, heart and skin. The alleged mechanisms for the induction of tolerance in these models in vivo are related to the production of regulatory T cells as well as the induction of tolerogenic DCs mediated through the expression of IDO (indoleamine 2,3-dioxygenase).<sup>28</sup> The inducing tolerance in vivo depends on being able to infuse expanded MSCs before the transplant, with (or without) small doses immunosuppressants such as mycophenolic acid.<sup>28</sup> The usefulness of MSCs has not only been demonstrated in transplants but recently data to this effect have also emerged from autoimmunity models.

#### *Manipulation of Regulatory Cells to Induce Transplant Tolerance*

From what has been mentioned already, the great therapeutic potential of regulatory lymphocyte populations for the control of allogeneic rejection is evident. Several techniques have been described that prevent effectively graft rejection in rodent models by inducing Treg

and Tr1 cells in the context of allogeneic reactions. However, the application of these findings to controlling alloeneic rejection in humans still faces several problems. First, it must be determined which population of regulatory lymphocytes is the most suitable. While Treg cells have phenotypic characteristics that allow them to be enriched relatively easily, the current absence of an exclusive marker for this population in humans makes it almost impossible to obtain pure Treg populations. The presence in purified samples of small percentages of activated responding lymphocytes may raise important problems. Firstly, the protocols developed to expand Treg cells *in vitro* also induce the proliferation of contaminating effector T cells. Secondly, the fact that Treg cells mediation of suppression is antigen-independent, may produce a state of immunosuppression as an undesirable side effect. Finally, it should be taken into account that in clinical transplantation regulatory cells will be induced in the presence of various immunosuppressants necessary to guarantee the survival of the graft. One of the most active fields in relation to this is the search for immunosuppressive regimens that would facilitate the induction and/or maintenance of regulatory cells.<sup>29</sup>

Despite all these difficulties, several clinical trials in humans have been implemented using populations of both Treg and Tr1 for the prevention of allogeneic reactions. These trials are being carried out in the context of European and American Multicentre initiatives (RISET: Reprogramming the Immune System for Establishment of Tolerance and the ITN: ImmunoTolerance Network). Once the remaining issues are solved, it may be possible to isolate and expand T cells with higher suppressive activity that would allow the degree of immunosuppression and thereby the long-term adverse effects to be reduced, while maintaining a state of tolerance that would increase the survival of the allograft and the receptor. If this is not achievable, at least it will be possible to identify which transplant recipients have more or better-functioning Treg cells, indicators of a more effective state of tolerance, and so would need smaller doses of immunosuppressive drugs.

## CONCLUSION

Advances in the understanding of the immune response and the continuous appearance of new biological molecules have boosted the growing interest in transferring the knowledge concerning immune tolerance from experimental models to clinical transplantation. Many of the tolerance inducing manipulations explained in this chapter are currently being evaluated in clinical trials. The aim is to achieve an equilibrium through the manipulation of the cells involved in allograft rejection and those involved in immune tolerance. For this, Treg and dendritic cells seem to be the most promising candidates.

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## CHAPTER 5

# IMMUNOSUPPRESSION IN THE ERA OF BIOLOGICAL AGENTS

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**Abstract:** Immunosuppression is the mayor mechanism to prevent allograft rejection and to induce tolerance. Since the first solid organ transplant, the development of safe and effective immunosuppressive regimens was a constant over the last decades. A lot of immunosuppressants have been discovered, and today the immunosuppressive agents are classified in two broad groups: Xenobiotic immunosuppressants and biological immunosuppressants. Xenobiotics, like corticoids and calcineurin and mTOR inhibitors, mainly interfere with the intracellular molecular mechanisms of the various types of cells involved in the immune response and generally these immunosuppressants are used early on in the transplantation process to prevent rejection as well as in long-term maintenance therapy. On the other hand, target molecules of biological immunosuppressants are on the surface of these immunological cells and normally in clinical immunosuppressive protocols have been used as auxiliary agents of xenobiotics to prevent rejection as well as in the treatment of acute rejection. However, these xenobiotics and biological agents have multiple side effects; that is why there has been a search for new drugs to minimise these side effects and to improve patients' quality of life. In this way, new biological agents have been proposed as maintenance immunosuppressive agents. The majority of these new immunosuppressive agents are polyclonal or monoclonal antibodies and recently the so-called fusion proteins may be the start of a new era of biological immunosuppression for maintenance regimens.

## INTRODUCTION

In kidney transplants and in solid organ transplantation in general, two broad groups of immunosuppressive agents have been used: Xenobiotic immunosuppressants or small immunosuppressive molecules and biological immunosuppressants. While Xenobiotics mainly interfere with the intracellular molecular mechanisms of the various types of cells involved in the immune response, target molecules of biological agents are on the surface of these cells. Xenobiotics are used early on in the transplantation process to prevent rejection as well as in long-term maintenance therapy. On the other hand, biological agents have generally been used as auxiliary agents of xenobiotics in so-called induction protocols to prevent rejection as well as in the treatment of acute rejection. More recently, new biological agents have been proposed as maintenance immunosuppressive agents. The majority of biological agents are polyclonal or monoclonal antibodies and recently the so-called fusion proteins have also been added to the list.

The history of immunosuppression dates back to 1958, when radiation was used for the first time in a kidney transplant from a deceased donor, and the organ functioned for 32 days. In the nineteen-sixties, Azathioprine and corticoids were introduced. These two types of drugs were used together for some years in kidney transplantation, achieving survival rates after one year of 40%.

In 1972, Prof J.F. Borel discovered the immunosuppressive properties of cyclosporin A (CsA), a cyclic polypeptide consisting of 11 aminoacids that is obtained from *Tolypocladium inflatum* and related fungi and inhibits calcineurin. Clinical trials were started quickly and, in 1978, CsA commenced to be used in clinical practice, demonstrating its high efficacy for the prevention of rejection, as well as for dramatically improving in graft and patient survival.

The impact of CsA on the clinical practice of transplant medicine was spectacular. It has been used across the world and serves as the background therapy for current immunosuppressive regimens. In addition, it has enabled transplantations to be carried out of organs that were almost impossible before its introduction, including heart, liver, pancreas and lung.

However, it has multiple adverse effects; that is why there has been a search for new drugs to minimise these side effects and to improve patients' quality of life. Tacrolimus, a macrolide isolated from the fungi *Streptomyces tsukabaensis* and discovered in 1984, is nowadays the calcineurin inhibitor mostly used in transplantation. Another antimetabolite immunosuppressant, mycophenolate mofetil (MMF), is used in combination with CsA and corticoids, and can be used instead of azathioprine to prevent acute rejection in renal transplantation. These two immunosuppressants, mentioned above, were introduced in clinical practice in the nineteen-nineties.

Other recently introduced xenobiotic immunosuppressive drugs are sirolimus, also known as rapamycin, a lipophilic macrolide obtained by the fermentation of actinomycete *Streptomyces hygroscopicus*, and everolimus, derived from the former by chemical modification to improve absorption.

On the other hand, as we will discuss later, biological agents are used in clinical practice mainly to prevent but some of them also to treat severe acute rejection. Polyclonal antibodies have been in use since the nineteen-seventies, and monoclonal antibodies, such as IL-2 anti-receptors and other new generation compounds such as Alemtuzumab (Campath-1), and belatacept, are being introduced and seem very promising.

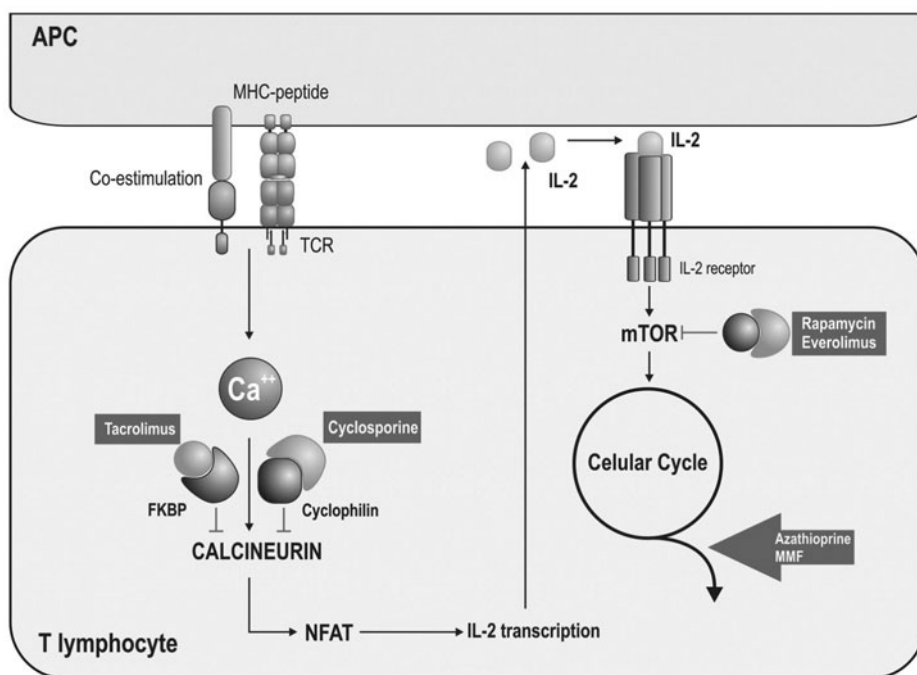
## XENOBIOTIC IMMUNOSUPPRESSANTS

The most commonly used xenobiotic immunosuppressants (Fig. 1) have been: Steroids, present in the majority of the treatment regimens to date; antimetabolites; calcineurin inhibitors (CsA and tacrolimus); and more recently m-TOR inhibitors. These immunosuppressants, depending on the regimen used and the characteristics of the patient, are always used in combination.

### Corticoids

Corticoids, prednisone and its active metabolite prednisolone are considered an important component in many immunosuppressive regimens and are commonly used as the first line treatment for acute rejection. These nonbiological immunosuppressants have a great variety of anti-inflammatory and immunomodulatory effects including: Stabilization of lysosomal membranes; inhibition of prostaglandin synthesis; reduction in the secretion of histamine and bradykinin; and decrease in capillary permeability, causing migration of lymphocytes from the vascular tree to the lymphoid tissue.

Corticoids enter the target cell and bind to glucocorticoid receptors. This binding gives rise to the translocation of the complex formed into the cell nucleus. In the nucleus, the transcription of certain genes coding for proinflammatory cytokines is affected by the inhibition of the nuclear activation factor B (NF- $\kappa$ B), among others, and by the binding



**Figure 1.** Mechanisms of action of xenobiotic immunosuppressants used in the prevention of transplant rejection. (FKBP: FK506 binding protein; NAFT: Nuclear Factor of Activated T cells; mTOR: the mammalian Target of Rapamycin; MMF: Mycophenolate Mofetil).

to the so-called glucocorticoid response elements, located in the promoter regions of the genes that control the production of cytokines such as IL-1, IL-2, IL-6, IFN- $\gamma$  and TNF- $\alpha$ .

These steroidal treatments have several side effects: On the one hand, metabolic effects such as diabetes, due to alterations in carbohydrate metabolism, changes in body fat distribution and loss of skeletal muscle proteins causing weakness. There is also fluid retention, as a consequence of the action of mineralcorticoids, with associated hypokalaemia and hypertension. Long-term glucocorticoid therapy leads to suppression of adrenal function and, eventually, to atrophy of the adrenal glands. Other side effects include psychosis, cataracts and glaucoma, and osteoporosis.

### **Antimetabolite Immunosuppressants**

Antimetabolite immunosuppressants include azathioprine, MMF and, more recently, mycophenolate sodium (MFS). Azathioprine<sup>1</sup> together with glucocorticoids was the basis of conventional immunosuppression from the beginning of transplantation until the mid nineteen-eighties. It is metabolised to 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG). These metabolites are incorporated in DNA, inhibiting its replication. They also interfere in the de novo synthesis of purines by producing thioinosinic acid. This effect is responsible for T-cell specificity by disrupting the pathways of purine synthesis. Recent studies indicate that azathioprine also interferes in the CD28 costimulatory pathway of alloreactive T cells. Specifically, the blocking of the CD28 costimulatory signal by azathioprine metabolites gives rise to apoptotic signals and deletion of the activated T cells.

The main side effect of azathioprine is dose-dependent bone marrow suppression, but occasionally it can cause liver damage and cholestatic jaundice. Multiple hypersensitivity reactions, seen in form of a rash, have also been described.

Mycophenolic acid is the active metabolite, produced in liver, of MMF and MFS.<sup>2</sup> This acid is an antipurine agent that inhibits the de novo synthesis of guanosine nucleotides, which are essential in the replication of DNA. T cells do not have salvage pathways to recover guanosine nucleotides, so blocking of the de novo synthesis by mycophenolic acid selectively inhibits the proliferation of this cell-type.

Potential uses of these immunosuppressants include the treatment of acute rejection in association with other drugs in order to strength baseline immunosuppression, reduction of CsA or tacrolimus dosage in patients with intolerance to these drugs (due to side effects), and early withdrawal of corticoids. Mycophenolic acid has several side effects: The most common is diarrhoea (dose dependant), but it can also cause nausea, sickness and abdominal pain. In addition, it may lead to bone marrow suppression and several studies have observed a high incidence of viral infections, in particular by cytomegalovirus (with infections by Candida and Herpes Simplex also being common), compared to placebo groups or patients treated with azathioprine.

The usefulness of this type of immunosuppressant has been demonstrated in the treatment of chronic nephrotoxicity associated with CsA, enabling dose reduction or even total withdrawal with no increase of rejection.

### **Immunosuppression by Calcineurin Inhibitors**

The calcineurin inhibitor drugs (CNI) are CsA<sup>3</sup> and tacrolimus.<sup>4</sup> CsA was started to be used before understanding their basic mechanism of action, namely, their capacity to inhibit calcineurin (Fig. 1). Both immunosuppressants are considered to be prodrugs since

they have to bind to intracellular proteins to convert to their active form: In the case of CsA the protein is cyclophilin and in the case of tacrolimus it is immunophilin FKBP12. These molecular complexes inhibit calcineurin, by preventing the entry of NFAT (Nuclear Factor of Activated T cells) to the nucleus and start the transcription of various cytokines. They also inhibit the transcription of c-jun genes and block the translocation of NF- $\kappa$ B to the nucleus, thereby avoiding the transcription of cytokines, such as IL-2, IL-3, IL-4, IL-5, IFN- $\gamma$ , TNF- $\alpha$  and the granulocyte macrophage colony-stimulating factor (GM-CSF).

These two molecules have been considered the cornerstone of immunosuppression in organ transplantation; while chemically very different, they target the same enzyme which largely explains the shared toxicities associated with the two drugs.

In the case of CsA, it is recommended that therapeutic levels of the drug in the plasma are achieved early (that is, within the 3-5 days post-transplant). The highest levels in plasma are obtained after 3-4 hours after administration, with between 60-70% bound to erythrocytes and the rest to plasma proteins. It is rapidly distributed to highly vascular organs and accumulates in adipose tissue. Since the absorption CsA is very different from one individual to another, the dose must be adjusted in each patient to achieve suitable plasma levels. Generally the only value that was measured was the trough level (i.e., the level obtained from a blood sample taken immediately before the next dose of immunosuppressant). However recent studies have demonstrated that the best optimisation of the dose is achieved on the basis of maximum levels or C2 (i.e., blood taken two hours after a dose of ciclosporin A), since these levels more accurately reflect the exposure of the patient to the drug.<sup>5</sup>

CsA has multiple and diverse side effects that include: Nephrotoxicity,<sup>6</sup> neurotoxicity, hepatotoxicity, diabetes, hyperlipidemia, high blood pressure, hirsutism, gingival hyperplasia, and the development of lymphoproliferative disorders and other cancers.<sup>7</sup> At present some of these side effects are thought to be potentiated to the simultaneous long-term use of steroids. The acute nephrotoxic effects of CsA are a consequence of vasoconstriction in the kidney. On the other hand, chronic nephrotoxicity is probably caused by two mechanisms: Sustained vasoconstriction in the kidney (inducing ischemia), and induction of fibrogenic growth factors. Histologically, these processes result in obliterative vasculopathy (mainly vascular hyalinosis) and interstitial fibrosis.

Tacrolimus must not be combined with ciclosporin, since the side effects would be enhanced. With respect to the administration of tacrolimus trough levels are representative of drug exposure, although, to avoid the levels monitored being distorted by different rates of absorption of the drug, it is important that the patient always receives it in the same conditions, after fasting or after food. Accordingly, it is recommended that it be administered either 1 hour before or 2 hours after meals.

The diverse side effects of tacrolimus are practically identical to those of CsA: Nephrotoxicity, carbohydrate intolerance (more frequent than with CsA), neurotoxicity (often dose-dependent) and the possible development of lymphoproliferative disorders and primary cancer.<sup>7</sup>

### **Immunosuppression by m-TOR Inhibitors**

The inhibitors known as m-TORi, standing for mammalian target of rapamycin are so-called due to their ability to disrupt production of this enzyme. Rapamycin,<sup>8</sup> also known as sirolimus, and everolimus<sup>9</sup> are macrolides that share the same intracellular receptor as the immunosuppressant tacrolimus, FKBP-12 (Fig. 1). The FKBP/immunosuppressant

complex inhibits the TOR protein. The effect of m-TOR inhibitors is to block the action of IL-2, by disrupting biochemical processes that take place in the cell cycle of the target cell, preventing its proliferation. In short these are: Inhibition of S6 kinase (p70S6k), involved in important processes of the cell division cycle; repression of Bcl-2 transcription, a protein that protects cells from apoptosis; and prevention of CD28 activation.

Thus, m-TOR inhibitors block cell proliferation by preventing the binding of growth factors to their receptors in the cell, which in the case of lymphocytes is IL-2, but this is also valid for other cell types which explain the pleomorphic effects of these immunosuppressive agents.

Rapamycin and everolimus have the same side effects, which can be classified as: Metabolic, with increase in serum cholesterol and triglycerides and decrease of serum uric acid levels; haematological, with suppression and decrease of leukocytes, erythrocytes and platelets; dermatological, with appearance of acne and lip ulcers (herpes simplex); and others, related to the inhibition of growth factors. The advantage of these immunosuppressants is that they do not directly cause nephrotoxicity, neurotoxicity or diabetes, at least at usual doses (Table 1).

## BIOLOGICAL IMMUNOSUPPRESSANTS

### Polyclonal Antibodies

The first biological immunosuppressants used in human therapeutics, initially referred to as anti-lymphocyte sera, are currently called polyclonal antibodies, since they contain a mixture of antibodies against various lymphocyte surface molecules. They are obtained by injection of lymphocytes or thymocytes into animals, mainly rabbits, triggering an antibody response from which the IgG fraction is extracted, then purified to be used in human therapy. These are commonly known as anti-thymocyte globulin (ATG).

The rationale behind their design and use was their powerful ability to destroy the lymphocytes that they target. Severe lymphopenia can be induced by a single injection. The binding of these antibodies to lymphocyte surface molecules triggers complement-mediated lyses. However ATG also induces opsonisation and phagocytosis of lymphocytes by macrophages, and they have been shown to be able to induce lymphocyte apoptosis *in vitro* and *in vivo*. In addition, it has been demonstrated that *in vitro* they induce the expansion of regulatory T cells, which are able to suppress lymphocyte response in mixed lymphocyte cultures,<sup>10</sup> as well as induce apoptosis of dendritic cells,<sup>11</sup> granting them immunomodulatory properties of potential interest for tolerogenic regimens in human clinical practice. ATG contain a mixture of antibodies against T-lymphocyte surface antigens involved with immune response, cell adhesion and transport, and heterogeneous response pathways, as well as antibodies directed against B-cell molecules at all stages of maturation and against antigens of plasmatic cells, which is relevant for the treatment of antibody-mediated rejection.

### Therapeutic Use

Anti-thymocyte globulins are used in the treatment and prevention of acute rejection, and more recently in tolerance induction, regimens for patients with high risks of developing post-transplant renal insufficiency and in strategies for decreasing the use of steroids and calcineurin inhibitors.



**Table 1.** Summary of the characteristics and functions of xenobiotic immunosuppressants

Immunosuppressive Drug		Characteristics	Function	Adverse Effects
Prednisone and Prednisolone	Corticoids	<ul style="list-style-type: none"><li>• First line in acute rejection</li><li>• Anti-inflammatory and immunomodulatory</li></ul>	<ul style="list-style-type: none"><li>• Inhibition of proinflammatory cytokines</li><li>• Effects on cellular traffic</li></ul>	<ul style="list-style-type: none"><li>• Diabetes</li><li>• Loss of skeletal muscle proteins</li><li>• Hypertension</li></ul>
Azathioprine	Antimetabolite	<ul style="list-style-type: none"><li>• Active metabolite 6-mercaptopurine</li><li>• Used initially with corticosteroids in the first transplant protocols</li></ul>	<ul style="list-style-type: none"><li>• Inhibition of de novo purine synthesis</li></ul>	<ul style="list-style-type: none"><li>• Bone marrow depression</li><li>• Rarely hepatotoxicity</li><li>• Hypersensitivity reactions</li></ul>
Mycophenolic acid	Antimetabolite	<ul style="list-style-type: none"><li>• Derivative of mycophenolate mofetil and mycophenolate sodium</li><li>• Second-line drug in intolerant to cyclosporine and tacrolimus</li></ul>	<ul style="list-style-type: none"><li>• Blocking the synthesis of guanine</li></ul>	<ul style="list-style-type: none"><li>• Increased viral infections by cytomegalovirus</li><li>• Diarrhea and nausea</li><li>• Bone marrow suppression sometimes</li></ul>
Cyclosporin A	Calcineurin Inhibitor	<ul style="list-style-type: none"><li>• Binding to Cyclophilin</li><li>• Cornerstone of many immunosuppressive regimens, but many adverse effects</li></ul>	<ul style="list-style-type: none"><li>• Inhibition of transcription of proinflammatory cytokines</li></ul>	<ul style="list-style-type: none"><li>• Nephrotoxicity</li><li>• Neurotoxicity and hepatotoxicity</li><li>• Hyperlipidemia</li><li>• Development of lymphoproliferative disorders</li></ul>
Tacrolimus	Calcineurin Inhibitor	<ul style="list-style-type: none"><li>• Binding to FKBP12</li><li>• More potent than cyclosporine A</li><li>• Biochemically unrelated to cyclosporine A, but with same effects</li></ul>	<ul style="list-style-type: none"><li>• Same effects as cyclosporine A</li></ul>	<ul style="list-style-type: none"><li>• Posttransplant diabetes mellitus</li><li>• Similar adverse effects to cyclosporin</li></ul>
Rapamycin	mTOR Inhibitor	<ul style="list-style-type: none"><li>• Similar structure to tacrolimus</li><li>• Intracellular binding to FKBP</li><li>• Affects to calcium-independent events</li></ul>	<ul style="list-style-type: none"><li>• Inhibition of the action of IL-2</li><li>• Cell cycle block</li></ul>	<ul style="list-style-type: none"><li>• Metabolic, hematological and dermatological</li><li>• Less nephrotoxicity and neurotoxicity than anti-calcineurin</li></ul>
Everolimus	mTOR Inhibitor	<ul style="list-style-type: none"><li>• Biochemical modification of the previous</li><li>• Greater absorption</li></ul>	<ul style="list-style-type: none"><li>• Same mechanism of action of rapamycin</li></ul>	<ul style="list-style-type: none"><li>• Same effects as rapamycin</li></ul>



Like the monoclonal antibody OKT3 (targeting CD3), ATG has been considered as rescue agent for corticoreistant episodes of rejection. A recent meta-analysis<sup>12</sup> demonstrated that for this purpose there were no significant differences between the two types of antibodies. On the other hand, ATG has been used as the first line treatment for rejection and this has advantages with respect to the classical use of steroids according to the same meta-analysis.

With the widening of the criteria for acceptance of marginal donors, also called Expanded Criteria Donors (ECDs), the use of ATG has increased. These organs are more susceptible to damage derived from ischemia-reperfusion injury and correspondingly are more antigenic and associated with a higher incidence of acute rejection.<sup>13</sup> Since ATG contains antibodies against adhesion molecules the potential of these agents for the prevention of delayed graft function (DGF) and in rejection prophylaxis, especially in cases of ECD transplants, has been investigated. The study published by Hammer et al<sup>14</sup> considered a classic, demonstrated that polyclonal antibodies decrease *rolling* and adhesion of polymorphonuclear leukocytes to the vascular bed in a nonhuman primate experimental model, compared to anti IL-2R antibodies. On the other hand, intraoperative use of ATG before reperfusion of the organ, as opposed to post-operative use, decreases the appearance of DGF and the length of hospital admission.<sup>15</sup> Following the same pretransplant administration regimen, thymoglobulin was shown to have advantages compared to basiliximab in the prevention of acute rejection in patients with high risk of acute rejection that were also treated with CsA, MMF and steroids.<sup>16</sup> Specifically, the incidence of rejection, as demonstrated by biopsy, fell significantly with the use of thymoglobulin, dropping to 16% compared to 25% with basiliximab, and this reduced the percentage of rejection episodes that required the use of polyclonal antibodies to 1.4%. Further, in renal transplantation of organs coming from older donors, the use of ATG or alemtuzumab has been associated with rejection rates of below 20%.<sup>17</sup> In dual kidney transplantation using suboptimal kidneys, polyclonal antibodies greatly reduce the incidence of rejection; however, in combination with sirolimus, MMF and steroids, it does not provide clear benefits compared to the triple therapy regime without induction.<sup>18</sup>

Another benefit of the use of antibodies in induction regimens may be a reduced need for other immunosuppressive agents such as steroids and calcineurin inhibitors thereby avoiding their well known side effects. However, the withdrawal of steroids in kidney transplantation over the last decade seems to have been associated with an increase in the incidence of acute and chronic rejection, and therefore with a greater risk of graft loss. The risk factors identified in steroid-sparing regimens have classically included the time of withdrawal, the age of the recipient, and the renal function, sensitization to HLA antigens. More recently, the associated immunosuppression has also been recognised to influence in the risk of rejection after withdrawal. That is why the first attempts to withdraw steroids were made in patients considered to be low risk and some time after the transplant. In the initial studies on withdrawal, in patients treated with CsA and MMF, it was shown that prior induction with polyclonal antibodies reduced the risk of rejection. The low incidence of rejection in current therapies, as well as the empirical concept that late withdrawal may disturb the immune balance, has encouraged attempts to withdraw steroids in the first few days after the transplant. Excellent results have been reported concerning steroid withdrawal in the first week after transplantation with induction based on rabbit ATG, with rejection rates below 15% four years after transplantation and with graft and patient survival rates of around 90% over this same

period.<sup>19</sup> The combination of tacrolimus and sirolimus with thymoglobulin makes it possible to withdraw steroids five days after transplantation and the appearance of rejections is further reduced to 10%.<sup>20</sup> In a study of a small number of patients treated with tacrolimus and MMF, monitored with a protocol for biopsy, steroids withdrawal did not seem to be associated with an increase in histological damage.<sup>21</sup> Moreover, a multivariate analysis of more than 300 patients, the use of thymoglobulin reduced the risk of rejection by approximately 40%.<sup>22</sup>

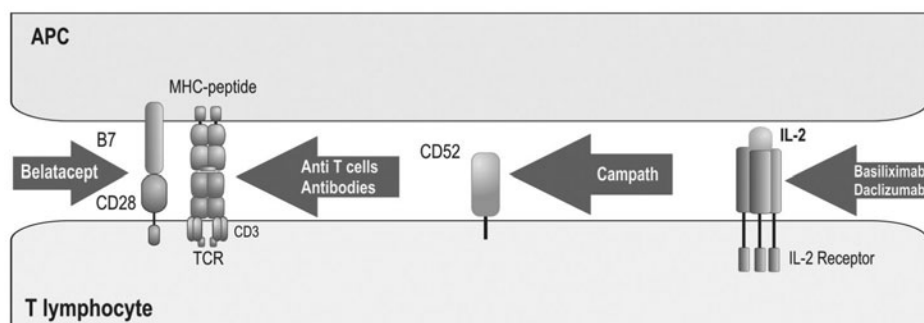
Due to their immunosuppressive capacity, ATG has also been tried to avoid the use of anti-calcineurin inhibitors. Initial noncontrolled trials that used rabbit ATG and MMF showed low incidence of rejection, but relatively high incidences of infection by cytomegalovirus and discontinuation due to side effects attributed to the high doses of MMF needed to compensate for the absence of calcineurin inhibitors.<sup>23,24</sup> Studies published in the last five years using ATG in combination with sirolimus, MMF and steroids report incidences of rejection of under 20%, improved renal function compared to with calcineurin inhibitors, and better histological response.<sup>25-27</sup> Nevertheless, a great limitation is the high rate of discontinuation due to side effects, attributable in most cases to the combined use of sirolimus and MMF, given the superimposing of the well known side effects of these two xenobiotic drugs. With respect to the aforementioned trials, a French study also attempted the withdrawal of steroids and this was achieved in approximately in 85% of the patients.<sup>28</sup>

Given the immunomodulatory effects of the ATG, it may be of interest in so-called tolerogenic regimens. In a pilot study, the use of high doses of ATG allowed the tacrolimus dose to be greatly decreased.<sup>28</sup> However, ATG in combination with sirolimus in maintenance monotherapy was associated with high incidence rejection rates.<sup>29</sup> On the other hand, the use of thymoglobulin, MMF and sirolimus, without steroids or calcineurin inhibitors, induces the expansion of regulatory T cells in the periphery and accumulation of these cells in the allograft accompanied by a donor-specific hyporesponsiveness,<sup>30</sup> which has been associated with improved renal function. All this can be expected to increase the interest in this type of immunosuppressive strategies.

### **Anti IL-2 Receptor Monoclonal Antibodies**

These antibodies were developed on the basis of that when T cells are activated after antigen recognition, the expression of IL-2 alpha chain of the receptor is induced (Fig. 2), and that resting T cells do not express this chain on their surface. That was why it was thought that monoclonal antibodies targeting the alpha chain might induce highly selective immunosuppression in only the clones of activated T cells.

At first murine monoclonal antibodies were used, but human xenosensitization against these heterologous proteins reduced their immunological effect. Consequently chimerical and humanized monoclonal antibodies, basiliximab and daclizumab, were developed. Basiliximab contains approximately 70% human protein, shows avidity for the IL-2 receptor similar to the murine antibody, and has a half-life of 9 days. By comparison, daclizumab is a humanized antibody that is 90% human protein; it has lower avidity for the receptor but its half-life is about 20 days, similar to human immunoglobulins. These characteristics entail two different administration strategies. Basiliximab is given on the day of the transplant and four days later, while daclizumab is administered on the day of the transplant and every other week, with a total of five doses. Both antibodies are used for rejection prevention and not effective for the treatment of



**Figure 2.** Schematic representation of the various therapeutic targets of the monoclonal antibodies used in solid organ transplantation.

rejection. It should be noted that their use has declined in favour of polyclonal antibodies. The first experiments with these antibodies showed that together with the therapies based on CNIs and steroids 10 to 15 years ago, and before the introduction of MMF, they were capable of reducing the incidence of rejection, with excellent tolerance levels and with no associated increase in opportunistic infections. More recently, they have been tested to help avoid or reduce the doses of anti-calcineurin inhibitors or steroids.<sup>31</sup> The use of daclizumab with mycophenolate and steroids in a CNI-free strategy was found to be associated with an incidence of rejection of 50%,<sup>32</sup> motivating a second study in which ciclosporin was added in small doses, and comparing temporary use and withdrawal. This early withdrawal regimen resulted in rejection rates of up to 40%, so it was concluded that the most reliable regimen should be based on the use of MMF with low doses of anti-calcineurin inhibitor and steroids. The Symphony study followed, in which the use of ciclosporin at conventional levels, were compared with low doses of tacrolimus, CsA or sirolimus in combination with mycophenolate and under daclizumab induction.<sup>33</sup> This study showed that the group treated with low doses of tacrolimus had better outcome in terms of low incidence of acute rejection (12%), better renal function and improved survival rates one year after the transplant.

As with polyclonal antibodies, basiliximab has been tested as a steroid-sparing agent. A recent study compared the total absence of steroids with their early withdrawal, one week after transplantation, at conventional levels in combination with CsA, MMF and induction with basiliximab.<sup>34</sup> In this study, in the two steroid-sparing groups, rejection rates were above 20%, although with no impact on renal function that had been the main objective of the study.

### Alemtuzumab (Campath)

Initially used for the treatment of leukaemia, alemtuzumab is an antibody that targets CD52 (Fig. 2) expressed in T and B lymphocytes. Despite not being indicated for use in transplantation, it has been increasingly employed across the United States due to its strong immunosuppressive effects and low cost in comparison with the previously described antibodies. Normally given in two doses, alemtuzumab induces deep and long-lasting T-cell depletion, reduces the number of CD4+ T cells recently emigrated

from the thymus and increases the ratio between regulatory and effector T cells, although it has also been associated with the appearance of acute rejection mediated by donor-specific antibodies.<sup>35</sup> Various analyses based on registries and comparative studies have reported a variety of results in terms of the superiority of this antibody compared to those described previously, in terms of the appearance of rejection and of graft survival.

### **Belatacept**

One of the concepts of transplant immunology is that the first signal of lymphocyte recognition of the antigenic peptide, presented in the context of the major histocompatibility complex by the antigen-presenting cell, must be accompanied by a second costimulatory signal. There are several costimulatory signals among which the importance of the interaction between CD80 and CD86 molecules, in the antigen-presenting cells, and the CD28 molecule in the T cell (Fig. 2) is well known. After antigen recognition, such interaction triggers T-cell activation and the secretion of cytokines. On the other hand, the CTLA4 molecule, that is expressed on T cells and also binds to CD80 and CD86 but has an opposite effect to CD 28, prevents T-cell activation. The lack of CTLA4 has been associated with autoimmune diseases in animals. The understanding of the regulatory mechanism of the costimulatory signals mediated by CD28-CD80/86, led to the design of the fusion protein CTLA4-Ig, which has an extracellular domain for CTLA4 while the intracellular domain substitutes the fragment Fc of IgG1. This fusion protein, called abatacept, was shown to be effective in murine transplant models and against autoimmune disorders. However it was not effective in preclinical transplants models in nonhuman primates. For this reason changes were made to the abatacept molecule, substituting the aminoacid leucine in position 104 for glutamate and the aminoacid alanine in position 29 for tyrosine (LEA29Y). This molecular change, producing the second generation drug belatacept, doubled the avidity for CD80 and increased its avidity for CD86 four-fold, while its capacity to inhibit T-cell activation increased ten-fold, and the modified molecule was effective for the rejection prevention in a transplant model in nonhuman primates.<sup>36</sup> These experimental results motivated a Phase II study that compared belatacept in combination with basiliximab, steroids and MMF, with a regimen in which the biological agent was replaced by CsA. This non-inferiority trial showed a similar incidence of acute rejection demonstrated by biopsy (19% with belatacept vs. 18% with CsA), better renal function and lower incidence of chronic nephropathy with belatacept in comparison to CsA.<sup>37</sup> On the basis of these promising results, two Phase III renal transplant trials in patients receiving organs from extended criteria donors (ECD) and conventional donors are being undertaken. It should be highlighted that, for the first time in the history of transplant immunosuppression, a biological agent is being used in long-term maintenance regimens, combined with MMF and steroids with no anti-calcineurin inhibitors, with stable renal function with no appearance of late rejection episodes.

### **Alefacept**

This is a LFA3-Ig and, previously used in the treatment of psoriasis, it is currently being used in the Phase II transplantation trials.

## CONCLUSION

To conclude we can state that biological immunosuppressants have been used hand-in-hand with xenobiotic drugs in recent decades in an attempt to improve the initial outcomes in the first months after transplantation. Currently, trials are being undertaken to refine existing regimens. In particular, the introduction of fusion proteins may be the start of a new era of biological immunosuppression for maintenance regimens.

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## CHAPTER 6

# TRANSGENIC ORGANS AND XENOTRANSPLANTS

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**Abstract:** Advances in immunosuppressive treatments reached in the last decades of the 20th century have made solid organ transplantation the treatment of choice for cases of irreversible organ failure. However, the availability of human cadaver organs is limited and the demand for transplants is still on the rise. Also, there is a recognised lack of cells and human tissues for generalised use in transplantation for the treatment of diseases that are characterised by failure of specialised cells (such as pancreatic cells to cure diabetes). Xenotransplantation, which is the transplant of cells, tissues or organs from other species, became the focus of attention in the nineteen-nineties as a solution to the lack of organs and tissues for transplantation. Previous clinical studies using nonhuman primates produced poor outcomes (survival from days to a few months) and confirmed the difficulty of obtaining organs from these species. Since then, progress in xenotransplantation has been slow and still now various immunological and non-immunological barriers need to be overcome. These barriers are reviewed in this chapter and the various approaches explored to date to overcome them, in particular those based on the genetic modification of pigs. Also, cell transplant studies such as those of pancreatic islets in monkeys have led to even more hopeful results. The range of possibilities offered by this technology will be unlimited, making it possible for xenotransplantation to be a clinical reality in a not very distant future.

## INTRODUCTION

Advances in immunosuppressive treatments reached in the last decades of the 20th century have made solid organ transplantation the treatment of choice for cases of irreversible organ failure. However, the availability of human cadaver organs is limited



and the demand for transplants is still on the rise. In Spain, the world leader in cadaver organ donation, the greatest achievement has been to avoid a year-on-year growth in waiting lists, as occurs in most countries. It has not been possible to reduce them, despite the notable increase of live donor transplantation; rather these have only served to slow the growth of waiting lists, not to mention the risks of live donor transplantation for the donor. Also, there is a recognised lack of cells and human tissues for generalised use in transplantation for the treatment of diseases that are characterised by failure of specialised cells (such as pancreatic cells to cure diabetes).

Xenotransplantation, which is the transplant of cells, tissues or organs from other species, became the focus of attention in the nineteen-nineties as a solution to the lack of organs and tissues for transplantation. At that point, after the production of the first transgenic pigs, this species was selected as the best source of organs and xenogeneic tissues.<sup>1-3</sup> Previous clinical studies using nonhuman primates produced poor outcomes (xenograft survival from days to a few months) and confirmed the difficulty of obtaining organs from these species. Moreover, the risk to public health of using the organs from nonhuman primates was quite high, as vividly demonstrated by the AIDS epidemic. On the other hand, pigs are domesticated animals, which produce large litters, and that, apart from their interest in the food industry, have also had some medical uses (production of insulin, heart valves). Finally, they combine a physiology and anatomy that is similar to primates and they can be genetically modified. Thus, it is not surprising that the possibility of “humanising” pig organs and tissues should be talked about with a view to using them in clinical practice.

Since then, significant advances have been made. The most well studied pig organs for their potential for xenotransplantation are the kidney and heart, followed by the lung and liver. Indeed, the liver is considered as the potential bridge for allotransplantation, either through transplant or by *ex vivo* perfusion. Research is also being carried out on cell xenografts of pancreatic islets, hepatocytes, chondrocytes and neural cells, among others. Despite the great impact that their clinical application would represent, progress in xenotransplantation has been slow, mainly due to technological problems. A decade later, we are still facing various barriers that prevent the clinical application of pig organs, tissues and cells. These barriers are reviewed below and the various approaches explored to date to overcome them, in particular those based on the genetic modification of pigs, are described.

## **OBSTACLES FOR CLINICAL XENOTRANSPLANTATION**

### **Immunological Barriers**

The main obstacle for xenotransplantation to be used in clinical practice is the strong immune response caused by the pig organ in the recipient.<sup>3</sup> Cell, tissue and organ xenografts are subject to a variety of rejection mechanisms that include humoral and cellular immune responses. The cellular immune response seems to play a key role in the rejection of cell grafts, such as hepatocytes and pancreatic islets, while rejection of vascularised organs mainly involves humoral immunological mediators. Various types of rejection have been described in solid organ xenotransplantation, depending on the time elapsed following the transplant and the immune elements involved: Hyperacute, acute humoral and acute cellular rejection.<sup>1-3</sup>



Hyperacute rejection (HAR) is the first-type to occur between minutes and hours after the xenotransplant. HAR is triggered by a humoral immune response in which xenoreactive natural antibodies (XNA) (already present in the recipient) are deposited on the endothelium of the xenograft, activating the complement system and causing fluid extravasation from the intravascular space into the interstitium (oedema).<sup>1-3</sup> This process also causes the activation of the coagulation cascade and thus thrombosis, ischemia and necrosis of the xenograft in a short period of time following the transplant. The main xenoepitope recognised by XNA is the disaccharide galactose- $\alpha$ -1,3-galactose (Gal), widely expressed in pig tissues and synthesised by the  $\alpha$ 1,3-galactosyltransferase ( $\alpha$ 1,3-GT) enzyme. Humans and Old World primates lack functional  $\alpha$ 1,3-GT and have high titre of anti-Gal antibodies.

Acute humoral xenograft rejection (AHXR), also known as acute vascular rejection or delayed xenograft rejection occurs in a period of days to months after the transplant. It has not been possible to prevent it using currently available immunosuppression regimens.<sup>1-4</sup> AHXR shows notable similarity to HAR since it involves a strong humoral immune response with the participation of antibodies, the deposition of complement proteins and thrombosis in xenografts. However, in other respects AHXR differs significantly from HAR, especially in the origin of the antibodies responsible for rejection. In this case, it involves a response in which anti-Gal antibodies participate, but it also includes antibodies targeting other epitopes. Further, AHXR is characterised by the presence of innate immunity cell infiltrates (NK cells and macrophages) and an activation of endothelial cells that promotes intravascular thrombosis and fibrin deposition.

Acute cellular rejection occurs some days after the transplant and is a response mediated mainly by T cells against the donor antigens. The activation of these cells during xenotransplant rejection is mediated by a primary signal through the T receptor and secondary costimulatory signals conserved across species.<sup>1</sup> Currently, it is thought that it is possible to control the cellular immune response against the xenotransplant using the various immunosuppressive protocols currently available, since no failure of pig xenograft caused by this type of rejection has been demonstrated. However, this point cannot be properly judged until AHXR can be prevented in an effective and systematic way.

Avascular tissue and xenogeneic cells are also subject to rejection of the graft, similar to the process that occurs in solid organ rejection, but without the vascular component and with special features for each cell and tissue.<sup>5</sup> Pancreatic islets have drawn great attention due to their potential clinical application for the treatment of diabetes. First, adult pig beta cells do not express the Gal antigen, thus reducing the humoral component of rejection. Potent immunosuppressive protocols focussed on averting the activation of T cells have led to long-term survival of pig pancreatic islets in diabetic monkeys (>140 days).<sup>6</sup> The problem for the clinical application of cellular xenotransplantation lies in the fact that, while they present fewer immunological barriers than solid organs, they still require strong immunosuppression of the recipient that cannot be sustained indefinitely. In any case, the strategies developed for this type of transplant may be very useful for their application in other organs of tissues of interest, especially regarding genetic modification of the donor pig that can involve a wide variety of cell and tissue types. Therefore any progress made in this area has an impact that goes beyond xenografts, be it to cells, tissues or organs.

*Strategies to Prevent Hyperacute Rejection*

The various techniques have been developed to prevent HAR can be summarised in two groups: Those focused on modifying the xenograft, and those that use systemic treatments to alter the immune response of the recipient. The most refined methods are based on the genetic modification of pigs used as the source for organs, since they are less harmful for patients and their objective is to decrease the need for immunosuppression or other conditioning treatments. However, these are complex techniques and involve slow and expensive procedures. Systemic treatments, on the other hand, may help us to identify molecules or key rejection processes and to speed up the clinical application of xenotransplants. Specifically, the key roles of XNA and complement in HAR were confirmed by the effectiveness of antibody absorption by plasmapheresis and systemic complement inhibition to prevent this type of rejection.

The first approach to neutralise HAR by genetic engineering was the inhibition of complement activation through the expression of human complement regulatory proteins in transgenic pigs.<sup>7,8</sup> The reason for this was the notable restriction of the function of the complement regulatory proteins between species, which led to the suggestion that pig molecules were unable to control the activation of the human complement. Subsequent studies showed that pig complement regulatory proteins were also able to regulate, at least in part, the activity of the human complement.<sup>9</sup> This suggests that the benefit of the expression of the human complement proteins in pig cells is not only due to specificity, but also to the increase in expression of these proteins.

Initially in vitro studies demonstrated that the expression of human CD59 (hCD59) or CD55 (hDAF) in pig cells notably protects these cells from cytolysis triggered by the human serum. Ex-vivo perfusion experiments with human blood also demonstrated that kidneys and hearts from pigs transgenic for human CD59 functioned for longer than control animals. Later it also demonstrated the almost systematic prevention of HAR and longer survival rates of transgenic pig organs that expressed human complement inhibitors in transgenic pig-to-primate models.<sup>2,10</sup> However the use of organs from these transgenic pigs did not achieve full protection against humoral xenograft rejection, since all the organs were subject to AHXR sooner or later, the various immunosuppressive protocols being unable to modify this response (Tables 1, 2 and 3). This led research to focus on the production of pigs with genetic modifications that would reduce the reactivity of the organs to xenoantibodies present in human serum.

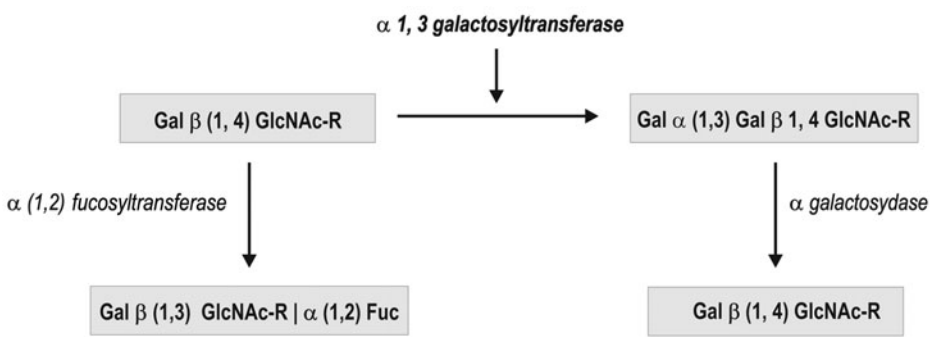
Before homologous recombination and “knockout” techniques were available in pigs, one of the most widely investigated strategies was based on the transgenic expression of human alpha 1,2-fucosyltransferase (H transferase, HT).<sup>11</sup> HT produced fucosyl residues (H antigen of the O blood group) that are universally tolerated (Fig. 1). HT was shown to compete efficiently with alpha1,3-GT for the same substrate, N-acetyl-lactosamine, preventing the transfer of the terminal galactose, the residue which gives rise to the production of the Gal antigen. The reduction in the expression of the Gal epitope in HT transgenic pig cells leads to a decrease in their reactivity with human antibodies and in cytolysis caused by human sera.<sup>11</sup> Further, the hearts of HT-transgenic mice have been shown to have higher survival rates after being perfused with human serum or transplanted to 1,3GT knockout mice, which produce anti-Gal antibodies. As HT and other competitive enzymes are not able to completely inhibit the expression of Gal epitopes, this technique was combined with the expression of human complement inhibitors. Thus, peripheral blood mononuclear cells and aortic

**Table 1.** Orthotopic kidney transplant of genetically modified pigs in cynomolgus monkey

First Author/Year	Treatment	Number of Cases	Mean Survival (Range)
Zaidi/1998	hDAF-tg + CsA + CyP + CS	7	13 days (6-35)
Cozzi/2000	hDAF-tg + CsA + CyP + CS + Spx + EPO	9	35.2 days (5-78)
Vangerow/2001	hDAF-tg + CsA + CyP + CS	4	11.5 days (9-15)
	hDAF-tg + CsA + CyP + CS + C1-INH	4	33.7 days (18-68)
Cozzi/2003	hDAF-tg + CsA + CyP + CS + Spx + EPO + MPS	10	24.1 days (2-51)
Cozzi/2003	hDAF-tg + CsA + CS + Spx + MPS + MTX	4	26.7 days (16-41)
Lam/2005	hDAF-tg + CsA + CyP + CS + MPS + GAS914	4	21.5 days (6-37)
	hDAF-tg + CsA + CyP + CS + MPS + GAS914 + sCR1	2	12.5 days (10, 15)
	hDAF-tg + CsA + CyP + CS + MPS + GAS914 before Tx + sCR1	4	24.7 days (10-37)

hDAF-tg, kidney of transgenic pig expressing hDAF; CsA, Cyclosporin A; CyP, cyclophosphamide; CS, corticosteroids; Spx, splenectomy; EPO, erythropoietin; C1-INH, C1 inhibitor; MPS, sodium mycophenolate; MTX, methotrexate; sCR1, soluble complement receptor-1; Tx, transplant.

endothelial cells of double transgenic pigs that co-express HT and hCD59 are better protected from lysis by human serum than controls or single transgenic cells for each of the genes.<sup>12</sup> In addition, double transgenic cells maintained their resistance to XNA and complement after being treated for 24 hours with porcine proinflammatory cytokines.<sup>12</sup> Despite these advances, these pigs were developed to be used in cellular xenotransplantation and the organs have not been transplanted to nonhuman primates, so it has not been possible to study their effectiveness in this experimental model in comparison to pig organs carrying other types of genetic modifications.



**Figure 1.** Biosynthesis of galactose α1,3 Galactose (Gal α1,3 Gal) and their inhibitors. The enzyme α1,3-galactosyltransferase adds galactose to the N-acetylglucosamine (Gal β1,4 GlcNAc) to form Gal α1,3 Gal. Transgenesis of the enzyme α1,2- fucosyltransferase produces a fucosylated structure (antigen H; blood group O), reducing the expression of Gal α1,3 Gal. Treatment with the enzyme α-galactosidase removes the terminal α-galactosyl residues of adjacent carbohydrates, preventing the expression of Gal α1,3 Gal.

**Table 2.** Orthotopic kidney transplants from genetically modified pigs to baboons

First Author/Year	Treatment	Number of Cases	Mean Survival (Range)
Lawson/1997	hDAF and hCD59-tg + CsA + CyP + CS	6	7.5 days (<10)
Diamond/1997	hDAF and hCD59-tg + CsA + CyP + CS + MPS	7	7.6 days (<15)
Cowan/2000	hDAF, hCD59 and HT low-tg + LMWH	6	4 days (3-5)
Buhler/2001	hDAF-tg + IA + CVF + ATG + CyP + CS + MMF + PGE + aCD154 + Spx + TmI + Heparin	3	28.7 days (28-29)
Cowan/2002	hDAF, hCD59 and HT low-tg + ATIII ± LMWH	4	5 days (4-6)
Ghanekar/2002	hDAF-tg + CsA + CyP + CS + GAS914 + RAD	2	28 days (20, 36)
	hDAF-tg + CsA + CS + GAS914 + RAD + RATS	4	23 days (20-26)
	hDAF-tg + CsA + CS + GAS914 + RATS	3	20 days (18-22)
Zhong/2003	hDAF-tg + CsA + CyP + CS + RAD low	4	18.5 days (4-40)
	hDAF-tg + CsA + CyP + CS + RAD low + GAS914 low	4	19.2 days (10-37)
	hDAF-tg + CsA + CyP + CS + RAD high + GAS914 high	4	14.5 days (9-25)
	hDAF-tg + CsA + CyP + CyP + CS + RAD high + GAS914 high	4	18.7 days (7-36)
Barth/2003	hDAF-tg + IA + CVF + ATG o Tmx + CyP + CS + MMF + PGE + aCD2 + aCD154 + Spx + TmKid	5	24.4 days (18-30)
Ashton-Chess/2003	hDAF-tg + IA + CsA + CyP + CS + MPS	4	6 days (5-9)
	hDAF-tg + CsA + CyP high + CS + MPS	4	9.2 days (7-12)
	hDAF-tg + IA + CsA + CyP high + CS + MPS	4	7 days (5-10)
Ashton-Chess/2004	hDAF-tg + CsA + CyP + CS + MPS	2	9 days (9)
	hDAF-tg + CsA + CyP alto + CS + MPS + Mx	4	8 days (6-10)
González Martín/2004	hDAF-tg + CsA + CyP + CS + MPS + GAS914	10	7 days (1-31)
	hDAF-tg + CsA + CyP + CS + GAS914 + FTY720	3	8 days (4-28)
	hDAF-tg + CsA + CS + GAS914 + FTY720 + Bas	3	8 days (3-13)
	hDAF-tg + CS + GAS914 + FTY720 + Bas + RAD	4	9 days (1-20)

*continued on next page*

**Table 2.** Continued

First Author/Year	Treatment	Number of Cases	Mean Survival (Range)
Ménoret/2004	hDAF and hCD59-tg	2	5.5 days (5, 6)
Yamada/2005	Gal KO + CVF + ATG + anti-CD2 + anti-CD154 + MPS + CS + Spx + Tmx ± WBI	3	29 days (20-34)
	Gal KO ± CVF + ATG + anti-CD154 + MPS + CS + Spx + Tmx ± WBI + VTL	6	34.1 days (4-68)
	Gal KO ± CVF + ATG + anti-CD154 + MPS + CS + Spx + Tmx ± WBI + VTL + TmKid	5	44.8 days (16-83)
Chen/2005	Gal KO + ATG + FK506 low + MMF + CS low	3	9.7 days (8-10)
	Gal KO + CVF + ATG + FK506 + MMF + CS	3	12.7 days (9-16)
Chen/2006	hDAF-tg + RATS o ATG + FK506 + CS + GAS914 o TPC + MMF	5	23.8 days (7-75)
	hDAF-tg + CVF + RATS o ATG + FK506 + CS + GAS914 o TPC + LF + DSG + aCD20	2	11 days (8, 14)

hDAF-tg, kidney of transgenic pig expressing hDAF only or other transgenes as indicated; Gal KO, knockout for  $\alpha$ 1,3-GT; CsA, cyclosporin A; CyP, cyclophosphamide; CS, corticosteroids; LMWH, low molecular weight heparin; ATIII, antithrombin III; MMF, mofetil mycophenolate; MPS, sodium mycophenolate; IA, immunoglobulin immunoabsorption; CVF, Cobra venom factor; PGE, prostacyclin PGE2; RAD, rapamycin derivative; ATG, antithymocyte globulin; RATS, rat anti-thymocyte serum; aCD154, anti-CD154 mAb; Mx, mitoxantrone; Bas, basiliximab or anti-IL2R mAb; Spx, splenectomy; Tmx, thymectomy; WBI, whole body irradiation; VTL, vascularised thymic lobes; TmKid, thymus-kidney; LF, LF15-0195; DSG, analogue of 15-deoxyspergualin; aCD20, anti-CD20 mAb.

The description of the process of nuclear transfer of a somatic cell into a germinal cell by the team led by Ian Wilmut in 1996 opened the door to the generation of “knockout” pigs. From that moment, several groups started the race to produce the first  $\alpha$ 1,3-GT deficient pigs. There was a concern that pigs produced by this technique would not be viable, but the first litters of  $\alpha$ 1,3-GT “knockout” pigs were healthy and developed well.<sup>13</sup> Subsequent studies showed survival times of up to 11 days for kidneys of  $\alpha$ 1,3-GT knockout pigs transplanted in baboons, meaning that the organs of these transgenic pigs were also protected against HAR,<sup>14</sup> as occurred in animals transgenic for human complement regulatory proteins. Despite this progress, the majority of human sera show reactivity towards pig cells that lack for  $\alpha$ 1,3-GT gene, suggesting the existence of antibodies that recognise other antigens apart from the Gal epitope.<sup>15</sup> Therefore, the introduction of a human complement inhibitor is still necessary to completely block human serum-mediated cytotoxicity.

Another carbohydrate in pigs of interest to prevent HAR in organ xenotransplantation is the antigen of the Type A blood group. As in the human, these antigens are present in some pigs and can be recognised by human antibodies directed against this blood-type. The reactivity of human serum IgM against this epitope was discovered in a kidney

**Table 3.** Orthotopic heart transplants from genetically modified pigs to baboons

First Author/Year	Treatment	Number of Cases	Mean Survival (Range)
Schmoeckel/1998 and Goddard/2002	hDAF-tg + CsA + CyP + CS + MMF	10	11.7 days (1-39)
Brenner/2005	hDAF-tg + CsA + CyP + CS + MPS	3	14.6 days (11-20)
Brandl/2005 and Brandl/2007	hDAF-tg + ATG + FK506 + CS + GAS914 low	2	5 days (1, 9)
	hDAF-tg + ATG + FK506 + CS + GAS914 high + aCD20	1	30 hours
	hDAF-tg + ATG + FK506 + CyP + CS + GAS914 high	3	13.3 days (1-25)
	hDAF-tg + ATG + FK506 + CyP + CS + GAS914 high + aHLA-DR	5	1.8 days (0, 2-4)

For these calculations the authors excluded cases of loss of organ due to technical failure and one case of HAR in the study of Goddard et al. hDAF-tg, kidney of transgenic pig expressing hDAF; CsA, cyclosporin A; CyP, cyclophosphamide; CS, corticosteroids; MMF, mofetil mycophenolate; MPS, sodium mycophenolate; aCD20, anti-CD20 mAb; aHLA-DR, anti-HLA-DR mAb.

from a Type A group pig whose kidneys were extracorporeally connected to a volunteer dialysis patient. Nevertheless, these antibodies should not be an obstacle to clinical xenotransplantation, since blood groups can be selected for the animals used as source of organs, in the same way as with allotransplantation.

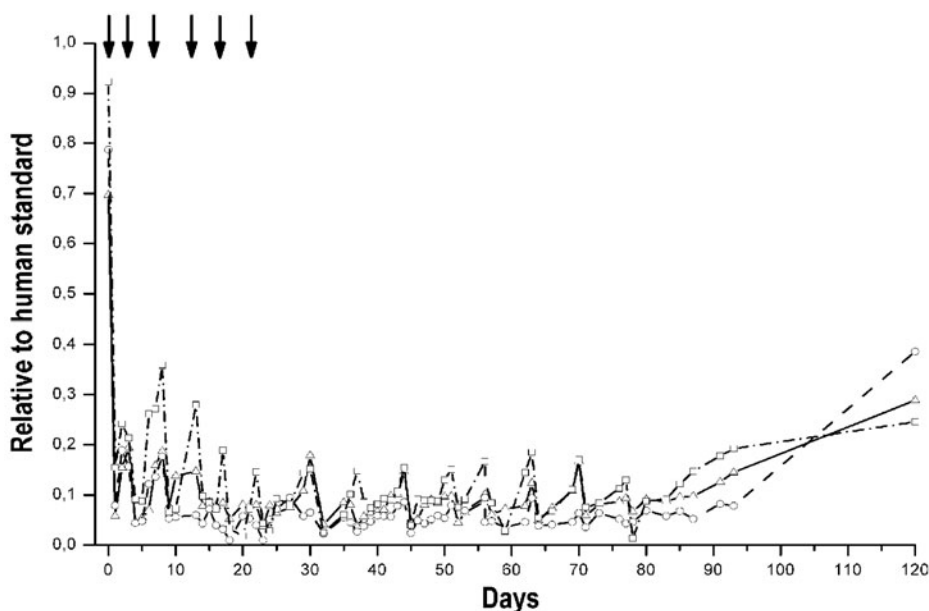
#### *Strategies to Prevent or Treat Acute Humoral and Cellular Xenograft Rejection*

The methods described above, alone or in combination, have successfully and routinely manage to prevent HAR. However the same strategies have not proved successful to avoid AHXR. The studies carried out to date suggest that this type of rejection is not caused by a single immunological element, but a collection of them. We will go on to describe research carried out so far into the control of humoral as well as cellular xenograft rejection. It is difficult to separate these two processes as they overlap in the time of progression and possibly also in the immunological response mechanisms. However, it should be highlighted that cellular rejection has not been an insurmountable obstacle and that the various immunosuppressive protocols investigated have managed to prevent this type of rejection systematically.

Most of the information currently available on preclinical xenotransplantation comes from studies using hDAF transgenic pigs as a source of organs and cynomolgus monkeys or baboons as recipients. Results of these studies are summarised in the Tables 1 to 3, focusing on orthotopic transplants of kidney and heart from genetically modified pigs, given their particular preclinical importance. The transgenic expression of hDAF, alone or in combination with hCD59, protects against HAR and confers some protection against HAXR.<sup>16,17</sup> One aspect that is worth highlighting is that the species of recipient primate also seems to influence the survival of the xenograft, survival rates being higher in cynomolgus monkeys than in baboons, perhaps due to the fact that the latter being a model that is closer to humans (Tables 1 and 2). Without immunosuppression AHXR

occurs 4 or 5 days after the transplant of hDAF transgenic organs in baboons,<sup>18</sup> there being no similar results from cynomolgus monkeys. The use of an immunosuppressive regimen that includes cyclophosphamide (CYP), cyclosporin (CsA) and corticosteroids (CS) increases the mean survival of the renal transplant recipient to 7.5 days in baboons and approximately 12 days in cynomolgus monkeys.<sup>2,10</sup> Parallel studies of orthotopic heart xenotransplants in baboons show mean survival rates of 12-15 days in similar conditions of immunosuppression. The addition of other immunosuppressants, such as rapamycin, mycophenolate mofetil or methotrexate, to the aforementioned protocol and performing splenectomy during the transplantation procedure, increased the mean survival rates for renal xenografts to up to 25-35 days in cynomolgus monkeys and to 9-20 days in baboons<sup>16,17</sup> (Tables 1 and 2). The temporary use of systemic complement inhibitors such as C1 inhibitor or soluble complement receptor 1 (sCR1, TP10), was also found to be effective, both in prolonging kidney xenograft survival and to revert AHXR, once diagnosed.<sup>19</sup> However, the toxicity of these products in the form of increased susceptibility to infections does not allow a long-term treatment with complement inhibitors.

Initially it was thought that anti-Gal antibodies also have an important role in AHXR, which led to the development of a series of polymers containing many Gal epitopes, following the failure of repeated immunoadsorption for neutralising these antibodies.<sup>18</sup> The most widely tested was GAS914, a trisaccharide composed of  $\alpha$ 1,3 Gal with a molecular weight of 500 kDa.<sup>20</sup> Using injections of GAS914 it is possible to continuously neutralise anti-Gal antibodies (Fig. 2). This also reduces the intensity



**Figure 2.** Continuous depletion of natural anti-Gal with GAS914 in baboons. The arrows indicate the days on which GAS914 was injected, with black corresponding to doses of 5 mg/kg and grey to 1 mg/kg. The levels of anti-Gal (circles), anti-Gal IgG (triangles) and porcine haemolytic (squares) antibodies are shown compared to a standard human serum, from a pool of 50 different human sera, which has been arbitrarily assigned a value of 1.



of the AHXR, although without improving xenograft survival even with the addition of potent immunosuppressive treatments.<sup>21</sup> The presence of antibodies in AHXR despite continuous depletion of anti-Gal antibodies in the blood suggested that the rejection was caused by antibodies directed against other pig epitopes. These results have later been confirmed with the production of  $\alpha 1,3$ -GT “knockout” pigs, that do not express the antigen Gal. Kidneys transplanted from these gal-deficient pigs into baboons have reached mean survival rates of 10 days under mild immunosuppression, and using higher-dose immunosuppression a mean of 29 days and a maximum of 34 days.<sup>15,22</sup> However these organs also suffer from AHXR mediated by antibodies other than anti-Gal that cannot be avoided with immunosuppressive treatments.

The best renal xenotransplantation results in baboons have been obtained using kidneys of  $\alpha 1,3$ -GT knockout pigs, in combination with protocols that use chimerism to promote graft tolerance.<sup>22</sup> Mean survival rates of 44.8 days, with a maximum of 83 days, have been attained by transplanting the pig kidney together with vascularised thymic lobe of the same animal, previously grafted under the renal capsule, with a conditioning regimen that included the temporary depletion of complement and T cells and maintenance with anti-CD154 monoclonal antibodies, mycophenolate mofetil and corticosteroids. The results represent an improvement compared to the mean and maximum survival rates of 24.4 and 30 days, respectively, achieved with similar experiments using kidneys from hDAF- transgenic pigs. This suggested that  $\alpha 1,3$ -GT knockout pigs offer advantages with respect to those that are transgenic for complement regulatory proteins in the AHXR, possibly due to the fact that the immune response is not amplified as shown in experimental models in rodents.<sup>5</sup> However it still remains unknown to what extent the combination of Gal deficiency by “knockout” with the transgenic expression of complement inhibitors would improve xenograft survival, compared to organs that have only one genetic modification.

A feature that has arisen in all the preclinical studies carried out to date is that treatments that inhibit the production of antibodies, such as high doses of cyclophosphamide or anti-CD154 monoclonal antibodies, do decrease or prevent AHXR. However, they all are very aggressive and lead to excessive immunosuppression, accompanied by severe side effects (gastrointestinal lesions, anaemia, infections, etc.) which in themselves jeopardise the life of the recipient. For this reason, the experiments carried out by the group led by Dr David Cooper are of great importance. These researchers performed a heterotopic heart xenograft transplantation in baboons from  $\alpha 1,3$ -GT knockout pigs, using immunosuppressive levels that were acceptable for clinical practice.<sup>23</sup> Most of these hearts were rejected with signs of AHXR or thrombotic microangiopathy. Nonetheless, they reached average and maximum survival times of 78 days and 6 months respectively, the longest survivals of porcine xenografts in nonhuman primates described to date.<sup>23</sup>

The thrombotic microangiopathy described in these experiments is considered to be another manifestation of AHXR, closely linked to the antibody-mediated response. Thus, recipients dying after long survival times due to causes not associated with rejection are found to have xenografts with minimal or non-existent pathological findings. For this reason, although it is not possible to completely rule out an effect of clotting incompatibilities between pigs and humans, as we will see in the following section that looks at the physiological barriers between these species, the control of the response mediated by anti- nonGal antibodies has become the biggest challenge for clinical xenotransplantation. Our group has preliminary data suggesting that these antibodies

are not directed against porcine proteins, but rather the targets, though not the Gal antigen, are also carbohydrates. Although it would be reasonable to expect that the antibodies are directed against many epitopes, their characterization would open the possibility of developing new treatments to prevent or treat AHXR. Given that one of the principal advantages of xenotransplantation is the possibility of modifying the donor organ, the solution for AHXR would come from genetically engineering the donor animal, to avoid the expression of those elements that cause an uncontrollable production of xenoantibodies.

Together with these measures intended to decrease the reactivity of anti-nonGal xenoantibodies, and potential clotting incompatibilities, other genetic modifications might decrease the immunogenicity of pig organs. Key potential targets are the immunological responses induced by the porcine cells, such as those mediated by the CD80-CD86/CD28 pathway, which is conserved across the swine-to-human species barrier. Specifically, CD86 expressed in porcine aortic endothelial cells provides strong costimulatory signals to human T and NK cells.<sup>1,24</sup> Porcine CD86, in contrast to its human counterpart, is expressed in a wide variety of cells and tissues, and the signal mediated by CD28 is resistant to immunosuppression by calcineurin inhibitors. Other elements which have a potential role in therapeutics are cytokines such as TNF $\alpha$  and  $\beta$ . The use of strategies that block the TNF may be useful in the development of xenografts resistant to AHXR, as has been demonstrated in rodent xenotransplantation models.<sup>25</sup> In general, the objective would be for these techniques to allow clinical xenotransplantation using the minimum possible level of immunosuppression in the recipient.

### Non-Immunological Barriers

#### *Physiology of the Xenograft*

It is well established that xenogeneic proteins such as porcine insulin can work correctly in humans. However, it is not clear whether xenografts are able to perform their functions in an environment other than that for which they have been genetically programmed, and, if so they are, for how long this functioning can be sustained. Pig kidneys have maintained the life of nonhuman primates for several months,<sup>16,22</sup> xenograft function apparently failing due to rejection rather than to the existence of physiological incompatibilities between the species. Recipients of  $\alpha$ 1,3-GT knockout kidneys required continuous treatment with human albumin to maintain protein levels within the normal range,<sup>22</sup> as a consequence of the proteinuria produced after the transplant and that continued throughout the three months that the xenograft survived. The clinical symptoms of the proteinuria (oedema) were different depending on the immunosuppression protocol used, suggesting that proteinuria was the consequence of the xenograft rejection, and not of a mutual physiological incompatibility. Furthermore, nonhuman primates transplanted with  $\alpha$ 1,3-GT knockout kidneys did not suffer from the anaemia previously described with the immunosuppressive protocols that included cyclophosphamide, confirming that it had been a consequence of the treatment toxicity rather than due to the inability of porcine erythropoietin to maintain erythropoiesis.

It has been confirmed that porcine hearts and kidneys are able of maintaining a similar physiology to human for long periods of time and they are candidates for the first solid organ clinical xenotransplants. In contrast, xenografts of other organs such as the lung and liver have not survived more than a few days, though even this has demonstrated that they can maintain the life of the recipient for short periods of time. Nevertheless,

some significant physiological differences can be expected between the donor and the recipient, especially in the case of the liver due to its complex metabolic system.

In the case of porcine heart and kidney, the existence of some long-term minor incompatibilities cannot be ruled out. An example of this is the clotting abnormalities described in AHXR. Currently it seems that these changes may be dependent of the deposit of xenoantibodies in the xenograft, but the existence of some kind of physiological incompatibility cannot be completely ruled out. In vitro, porcine cells have an inherent tendency to clot spontaneously in human plasma, an effect that seems to be dependent on some molecular incompatibilities between porcine and human blood-clotting regulators.<sup>26</sup> Specifically, porcine thrombomodulin (a key anticoagulant expressed by endothelial cells) hardly works in the human system.<sup>26</sup> If clotting abnormalities after xenotransplantation occurred independently of antibodies, the difficulties found in preventing and treating AHXR in porcine xenotransplants to nonhuman primates would be explained. Nevertheless, accumulated experience suggests that physiology should not be an insurmountable obstacle to clinical xenotransplantation of porcine organs. Also some methods are currently under investigation to solve the potential clotting incompatibilities between pigs and humans, including the genetic engineering of pig organs. If these problems were to persist once the problem of AHXR has been overcome, it is very likely that they could be also treated by the introduction of suitable human genes/proteins to the donor pig.

#### *Risk of Transmission of Infection from the Xenograft*

The success of organ xenotransplantation depends on the balance between the immunosuppressive treatment necessary to avoid rejection and the risk of opportunistic infections or cancer such treatment may cause. In the case of xenotransplantation, experimental data available to date suggest that more immunosuppression is required to prevent AHXR, and, therefore, that the theoretical risk of opportunistic infections is greater in xenotransplantation than in allotransplantation. Furthermore, the use of nonhuman cells, tissue or organs will increase the spectrum of opportunistic infections, since it will include diseases from the animal species used as a source of organs. The possibility that a new pathogen agent could be transferred to the recipient through xenotransplantation, and the possible passing of this to the general population, as happened with AIDS, is cause of concern among scientists and those responsible for Public Health.

The terms of “xenosism” and “xenozoonosis” have been proposed to describe infections produced by microorganisms of other animal species, that do not cause infection in humans in normal circumstances, but that might be transferred from a xenograft.<sup>27</sup> The probability of a specific microorganism of an animal species causing a disease in humans is unknown. In theory it may be fairly high in the cases of microorganisms that are zoonotic under normal conditions (for example, *Toxoplasma gondii*), similar to others that cause infections in allotransplantation (such as cytomegalovirus, CMV), and capable of infecting a wide spectrum of species (such as *Pneumocystis carinii*), as well as those microorganisms that can replicate in vitro in human cells. However, it is likely that xenosis, also known as xenozoonosis, caused by bacteria, fungi and parasites, arising from both common and species-specific pathogens, do not imply a particular risk for the recipient of the xenograft and even less for public health. The reason for this assertion is that this type of infections should be prevented in the animal that is to be the source of organs.

The production of animals in confined and isolated areas, from which only animals that are negative to all known pathogens are prospectively selected, can minimise the number of infections that these animals carry. Accordingly, it is important that strict protocols are established for clinical and microbiological assessment, using both immunocompetent and immunodepressed animals, to enable animals with any traces of infection to be detected. This applies, be they carriers of latent microorganisms, similar to those that cause infection in allotransplants, or of pathogens of other species. Removal of animals that are infected or are carriers of germs leads to the identification of the so-called specific pathogen free (SPF) animals, the use of which minimises the risk of transmission of both classical zoonosis and those arising from xenosis transmitted by the xenograft. In addition to these causes of exclusion for any animal, specific criteria can be included according to the organ to be transplanted. Thus, the presence of *Mycoplasma* sp. would rule out any animal as potential source for a lung xenograft, or the virus Coxsackie for heart xenografts. The ease of obtaining SPF pigs is another of the advantages of using this species as a source of organs, a process that logistically would be very difficult to carry it out with nonhuman primates.

The risk of transmission of the xenosis may be further reduced by the production of pigs totally free of germs (gnotobiotic). Although there are currently no facilities that allow the production of mammals in this state, their construction is totally feasible, with the high cost being the most significant disadvantage. However, gnotobiotic animals are less robust than those which have been subject to normal microbiological colonisation, and at present they do not seem to offer any advantages over SPF animals in terms of minimising the risk of transmission of xenosis. Therefore, the production of gnotobiotic pigs has been put on hold until clinical experimentation demonstrates its necessity.

With the production of SPF animals, the risk of transmission of diseases would be limited to some pathogens capable of producing latent infections in the source animal and caused by vertically transmitted viruses that cannot be prevented in the source animal by early weaning and/or caesarean birth. All species have viruses that persist within the host cells in a latent state. While the best known are the herpes viruses and retroviruses, these also include the hepatitis viruses, adenoviruses, rabies and pseudorabies viruses, reoviruses, and papovaviruses among others. It is still not known whether nonhuman latent viruses represent an infection and disease risk for humans. In vitro, it has been possible to infect human neurons with the porcine pseudorabies virus and to transmit various viruses between species in experimental models. Nevertheless, it is hard to believe that in the case of a species such as the pig, with which humans have been in contact for many thousands of years, there are many infections with capacity of being transferred from person to person that have not yet manifested themselves. On the other hand, the infection caused by Nipah virus in pig slaughterhouse workers in Asia, after contact with infected animals, and the recent epidemic of Severe Acute Respiratory Syndrome (SARS), caused by a coronavirus, transmitted through the consumption of exotic animals in China, are good examples of the ability these zoonoses to spread.

The potential re-activation of latent herpes virus infections after xenotransplantation of a porcine organ has been subject of particular attention in preclinical research on xenotransplantation. Three herpes viruses have been identified in swine: Porcine cytomegalovirus (pCMV), and porcine lymphotropic herpes virus 1- and 2- (PLHV-1, -2) which, in pigs that have been subject to bone marrow transplantation, are associated with a lymphoid proliferative syndrome similar to post-transplant proliferative disease (PTPD) seen in allotransplantation. Herpes viruses are species-specific so if infection occurs after

the xenotransplant, it should be restricted to the xenograft. The replication of pCMV has been described in porcine xenografts in nonhuman primates, causing an infection that damages porcine endothelial cells and tissues.<sup>28</sup> The elimination of pCMV from swine litters has been possible through early weaning of newborn animals, and the absence of pCMV in xenografts has been associated with the reduction of clotting disorders and improvement of survival rates of pig xenotransplants in nonhuman primates. Activation of PLHV-1 has not yet been demonstrated in any solid organ xenotransplant. However, unlike pCMV, this virus cannot be eliminated from source animals by early weaning of newborns, so remains a potential pathogen in porcine organ xenotransplantation.

Other infections that cannot be prevented using SPF pigs are those caused by vertically transmitted viruses. These include porcine endogenous retroviruses (PERV) which are viruses that have been permanently integrated into the genome of the host during the evolution of mammals, and are transmitted vertically from mother to offspring. Although they are not pathogens in the host, these retroviruses can be xenotropic, that is, capable of infecting other species. Two PERV have been known to have the capacity to infect human cells *in vitro*, which leads us to consider the possibility of recombination or complementation of xenograft endogenous retroviruses with viruses present in human tissues, and the potential risk of induction of tumours and immunodeficiencies caused by viruses.

Research undertaken to date, on humans in contact with living pig tissue, workers in pig slaughterhouses, human patients who have received transplants in contact with pigs and nonhuman primates who have received pig organs and severe immunosuppressive treatment, has not shown the existence of PERV replication in humans or nonhuman primates.<sup>29,30</sup> However work is ongoing to characterise PERV, to optimising systems for their detection, and to develop approaches to avoid or minimise the associated risks. On the one hand, certain families of miniature swine, “mini pigs”, that do not transmit PERV to human cells have been identified recently. On the other hand, transgenic techniques such as siRNA expression technique are being applied to inhibit the expression of PERV,<sup>31</sup> raising the prospect that this infection may be avoided by manipulating the animals to be used as sources for organs.

Given all this, on the basis of the currently available information, the risk of xenosis, also known as xenozoonosis, should not be an obstacle for clinical xenotransplantation of pig organs. In contrast to the situation existing a few years ago, risks have now been identified, investigated and tests have been developed that enable them to be assessed in preclinical and clinical studies. Although the possibility of an infection arising from the xenograft that would affect public health cannot be ruled out, the risk seems insignificant and avoidable by close supervision of the source animals and recipients of xenografts.

## CONCLUSION

Xenotransplantation has great potential to solve the problem of the lack of human tissues and organs for transplantation and continues to be a possible alternative to allotransplantation. Progress has been slow in research in this area due to technological problems, such as the difficulty of producing “knockout” pigs and the evaluation of xenozoonosis risks. However, the key tools have now been established and so the field can now develop much faster.

The main barrier to its clinical application is immune rejection, especially the humoral response triggered by vascularised xenografts. The identification of the key systems and molecules that are involved in the process of rejection, and the development of strategies to overcome them is just a matter of time. The use of porcine organs that have been subject to various genetic manipulations has already shown significant improvements in the xenotransplantation of organs to nonhuman primates. Cell transplant studies such as those of pancreatic islets in monkeys have led to even more hopeful results. Then the range of possibilities offered by this technology will be unlimited, making it possible for xenotransplantation to be a clinical reality in a not very distant future.

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## CHAPTER 7

# CELL AND TISSUE THERAPY IN REGENERATIVE MEDICINE

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**Abstract:** Cell therapy is one of the most promising future techniques in the medical arsenal for the repair of damaged or destroyed tissue. The diseases which cell therapy can target are very varied: Hormonal dysfunction, such as diabetes and growth hormone deficiency; neurodegenerative diseases, such as Parkinson's, Alzheimer's and Huntington's; and cardiovascular lesions, such as myocardial infarction, peripheral vascular ischaemia; as well as lesions in the cornea, skeletal muscle, skin, joints and bones etc. The objective of cell therapy is to restore the lost function rather than produce a new organ, which could cause duplicity and undesirable effects. Several resources of cells can be used to restore the damaged tissue, such as resident stem cells, multipotent adult progenitor cells or embryonic stem cells. Some cell therapies have been established and approved for clinical use, such as artificial skin derived from keratinocytes, derived from chondrocyte, cells of the corneal limbus or pancreatic islet transplantation. These therapies have had good results, although the scarcity of the starting material may represent a serious limitation. Other therapies under research, using pluripotent stem cells, have been modest so it is useful to review the protocols and try to improve the outcomes. In this chapter we will review the new advances made in this way.

## INTRODUCTION

Cell therapy is seen as one of the most promising future techniques in the medical arsenal for the repair of damaged or destroyed tissue. There are various approaches, including the use of both autologous (from the same individual) or allogeneic (from another individual of the same species) progenitors of adult or embryo stem cells and of

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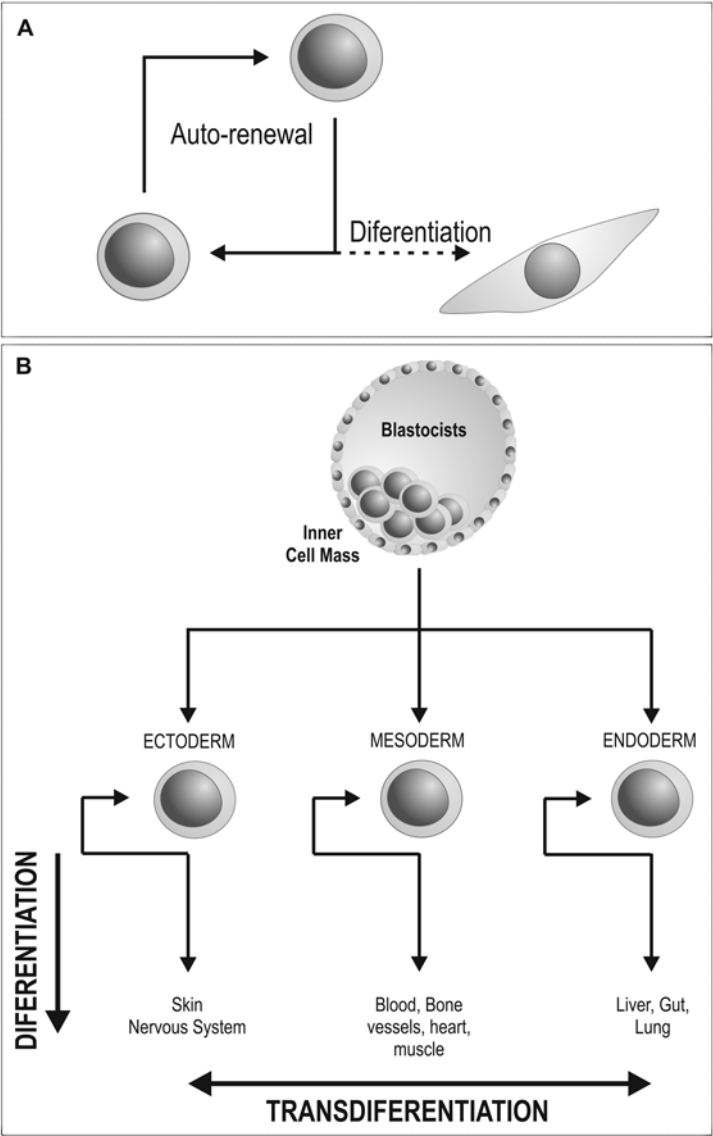
*Stem Cell Transplantation*, edited by Carlos López-Larrea, Antonio López-Vázquez and Beatriz Suárez-Álvarez. ©2012 Landes Bioscience and Springer Science+Business Media.

various trophic factors or cell pumps that produced them, with the objective of mobilising the stem cells residing in the host themselves and stimulating their proliferation and differentiation. The diseases which cell therapy can target are very varied: Hormonal dysfunction, such as diabetes and growth hormone deficiency; neurodegenerative diseases, such as Parkinson's, Alzheimer's and Huntington's; and cardiovascular lesions, such as myocardial infarction, peripheral vascular ischaemia; as well as lesions in the cornea, skeletal muscle, skin, joints and bones etc. Even though each condition has peculiar characteristics, the underlying principles of cell therapy remain much the same in these different applications, so it is very profitable to pursue a co-ordinated approach to research, development and clinical implementation in this field.

Cell therapy has similar indications to organ transplantation, but it is easier to carry out, since it does not require such a complex surgical procedure and, above all, because it could reach a much wider population. In Spain, the rate of organ donation, the highest in the world, is of around 34 per million people. With this rate, we could obtain 1.200 organs per year, a figure much lower than that of the potential recipients. As an example, the prevalences of Type I diabetes mellitus (insulin-dependent, and therefore susceptible to treatment by pancreas transplantation) or heart failure (which can be treated with a heart transplant) are of 100.000 and 200.000 respectively. Even with efficacy rates of 100%, it would not be possible to meet the needs of the new cases arising each year, around 2.000 and 3.500 respectively, and this demonstrates the need for therapeutic alternatives. The prevalence of neurodegenerative diseases is even higher (around 100.000 for Parkinson's disease and 600.000 for Alzheimer's), with 8.000 and 6.000 new cases respectively each year. Regenerative medicine exploits the therapeutic value of body's own growth factors and stem cells to repair cells, tissues and organs. It should be remembered that the aim is to restore the lost function rather than produce a new organ, which could cause duplicity and undesirable effects. For example, in the case of the heart, the presence of a new pacemaker could give rise to arrhythmias.

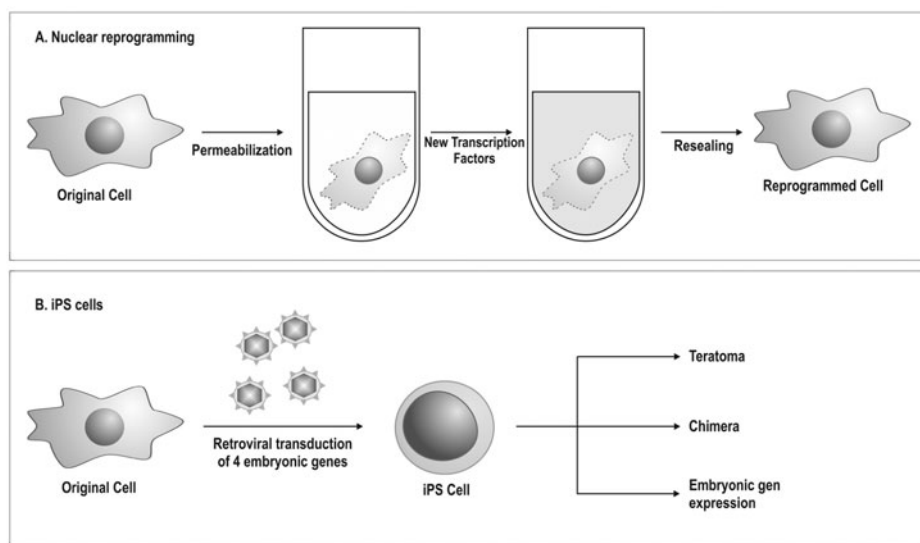
## CHARACTERISTICS AND TYPES OF STEM CELLS

Stem cells are able to divide, both to self-renew and to differentiate into specialised cells. This double function is achieved through a process of nonsymmetrical division in which one of the daughter cells remains undifferentiated and maintains the pool of stem cells, while the other one differentiates<sup>1,2</sup> (Fig. 1A). The cells obtained from a fertilised oocyte in the first divisions are totipotent, that is able to give rise to a complete embryo. After about four days the blastocyst is formed. At this stage, the cells of the inner cell mass (ICM, which will give rise to the embryo) start to specialise, forming three layers: The endoderm, the ectoderm and the mesoderm (Fig. 1B). The embryonic stem cells obtained from the inner cell mass are pluripotent, that is, able to differentiate into almost any type of cells of the three germ layers. Once the three layers are formed, the stem cells of each layer are multipotent, that is able to produce only a limited range of cell types depending of their location (for example, hematopoietic stem cells of the bone marrow can produce all the blood cell types). Finally, in many adult tissues stem cells are unipotent, able to produce only one type of cell (for example, the epidermis of the skin, the intestinal mucosa, the liver or skeletal muscle). In the adult, stem cells are normally located into niches with specific microenvironments which determine their behaviour.<sup>2</sup> From an orthodox point of view, the daughter cells undergo differentiation



**Figure 1.** A) Assymetric division of stem cells. B) The three germ layers of the embryo.

into a cell lineage of the same germ layer (vertical arrow in Fig. 1B). However, based on recent observations, it has been proposed, and this is very controversial, that under the pressure of an unusual environment (for example, tissue damage) the stem cells of a germ layer may also be able to produce mature stem cells that normally correspond to another layer, a process known as transdifferentiation (horizontal arrow in Fig. 1B). Some researchers hold the opinion that, since all cells are provided in the nucleus with all the genetic information, transdifferentiation would simply be the consequence of



**Figure 2.** A) Nuclear Reprogramming. Based on Collas,<sup>3</sup> 2003. B) Production of induced pluripotent stem cells. Based on Takahashi et al<sup>37</sup> 2007. Please note that the result is the same in both procedures, but the expression is achieved in a different way, with cell extracts that contain transcription factors (A) and with viruses that induce the expression of a gene (B), respectively. Reprogramming could also be achieved using cell extracts that work as trophic factors and produce selective demethylation of the genome.<sup>4</sup>

nuclear reprogramming (Fig. 2A) with the transcription of different genes caused by a change in transcription factors. There are some indications that such reprogramming could be achieved *in vitro* using nuclear and cytoplasmic extracts<sup>3</sup> or epigenetic techniques.<sup>4</sup> Recently, nuclear reprogramming has been achieved through the expression of certain genes using viral vectors (see induced pluripotent stem cells, iSP, below). For others, transdifferentiation requires prior dedifferentiation to earlier stages and subsequent differentiation along a different path. Finally, some scientists sustain that transdifferentiation does not really occur, and that the changes attributed to this process are really due to the fusion of the nucleus of the stem cell with a differentiated cell of a different germ layer.<sup>5</sup>

In practice, when we want to regenerate damaged tissue, we can choose between three different types of cells:

1. **Resident stem cells (RSC):** In the relevant tissue. Some types of tissues, such as the skin, the intestinal mucosa and the liver regenerate easily following this mechanism. If we could find a way to stimulate this mechanism in other tissues, for example, using the adequate trophic factors, this would be a very convenient therapeutic procedure, since the desired effect would be achieved without the need for surgical intervention. Alternatively, it would be possible to isolate the progenitors from the tissue of the recipients themselves, treating the cells *in vitro* to promote their expansion and re-implanting them in the desired location. This approach is the base of some established therapies (see below). Until recently it was thought that some types of tissue, including the heart and

the nervous system, did not contain resident stem cells and there they were “terminal”. However, it has been recently found that both the central nervous system<sup>6</sup> and the heart<sup>7,8</sup> do contain resident stem cells, although they are not able to, in practice, to regenerate the damaged tissue spontaneously. It seems possible, however, that they form neurospheres or cardiospheres that could be expanded in vitro and could potentially being re-implanted in the tissue. Another possible approach is to implant cells that produce trophic factors which would be able to stimulate resident stem cells and/or delay the death of damaged cells. This procedure is being investigated and is of great interest given its potential applications in relation to the nervous system.<sup>9</sup>

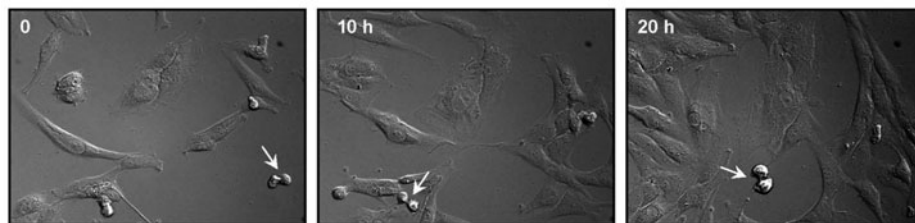
2. **Multipotent adult progenitor cells (MAPC):** When possible, these are an excellent option. A paradigm of this application is the transplant of bone marrow for the regeneration of blood cells, a well-established treatment for leukaemia. Recent data suggests that the plasticity of adult progenitor cells is greater than previously thought, since they have been found to generate cells or lineages that do not strictly correspond to their organ of origin. When adult progenitor cells of patients themselves are used, we avoid, on the one hand, the immunological problems and, on the other, many of the technical and legal problems, since the recipient and the donor are one and the same. To date, the cells used most frequently have been those derived from the bone marrow, since there is some proof of their pluripotentiality and their biosafety has been confirmed over the almost 50 years they have been used for the treatment of blood diseases (see below). Another cell-type that has aroused great interest is mesenchymal cells, which can be derived from the bone marrow as well as from other locations, and may have a greater pluripotential (see below). In addition, umbilical cord blood, which can be readily collected at birth and for which there are already specific banks, is not still widely exploited and may be of strategic interest.<sup>11</sup>
3. **Embryonic stem cells (ESC):** An approach based on using embryonic stem cells would be the most versatile since, given that they are pluripotent, the same cells and procedures could be used for different tissues. Other advantages include the relative ease with which they can be obtained, possibility of freezing, rapid and indefinite expansion retaining normal karyotypes. There are, however, several practical problems that have prevented their therapeutic use to date. One of them is immunogenicity and rejection, since in most cases allogeneic transplants would have to be used (though see also discussion of “nuclear transfer” and “induced stem cells” below). Another very serious problem is that of biosafety, since embryonic stem cells produce tumours (teratomas), which so far have proved impossible to prevent. Finally, despite the fact there has been great progress in the regulatory and social aspects (see below), there are still some ethical and legal problems and a little to no clinical experience which makes the use of ESCs difficult.

## ESTABLISHED THERAPIES

This is the term used to refer to cell therapy procedures that, given their use to date and proven therapeutic efficacy, have been approved by the Spanish Drug Agency and

can, therefore, be carried out without prior request for special permits (see below). Other therapeutic procedures with live cells have to be considered as clinical trials and require the corresponding permits. The approved therapies are listed below:

1. **Blood therapies**, which have been used for 50 years.
2. **Artificial skin derived from keratinocytes:** This technique, designed in collaboration by JL Jorcano (Madrid, Spain) and A Meana (Oviedo, Spain), has proven its usefulness in the treatment of major burns.<sup>12</sup> Basically it consists of obtaining a sample of keratinocytes from a healthy zone of the patient and expanding them to form sheets of skin in the laboratory, a process that takes around three weeks. Meanwhile and in order to avoid the fluid loss through the burned surface, a graft is carried out using cadaver skin. This tends to be rejected beyond 3 weeks but, before this occurs, it is replaced by autologous skin prepared from the keratinocytes of the patient. To date, the procedure has achieved good outcomes in a wide range of patients in various Spanish hospitals. The skin derived from keratinocytes is not of high quality, since it does not include skin adnexa such as hair follicles or sweat glands and may, at a later stage, be replaced by autografts.
3. **Autologous chondrocyte implantation in chondral defects:** Chondrocyte readily proliferate in vitro (Fig. 3) and there are already plenty of examples with regards to the treatment of articular lesions using autologous chondrocytes.<sup>13</sup> Cells are obtained from a non-affected zone within the articular cartilage and are grown, generally on collagen membranes, for around 3 weeks. Then, the surgeon places and secures the autologous chondrocytes on the damaged area of the articular cartilage. The procedure has been carried out, in knee lesions, in several hundred patients and results have been excellent.
4. **Cells of the corneal limbus:** In the corneal limbus there are corneal epithelial stem cells, which have been used to repair corneal ulcers.<sup>14</sup> The procedure consist of obtaining, in the surgical theatre, small fragments of the limbus, which are implanted during the same surgical session in the corneal ulcer. Given the limitations with regards to the amount of the starting material, the procedure is only applicable to relatively small lesions, and the success rate is not 100%. To solve these problems, it has been proposed that cells of the corneal limbus could be expanded in vitro before being implanted in lesions. This procedure, which should not be regarded an established therapy, is currently being investigated in several laboratories.



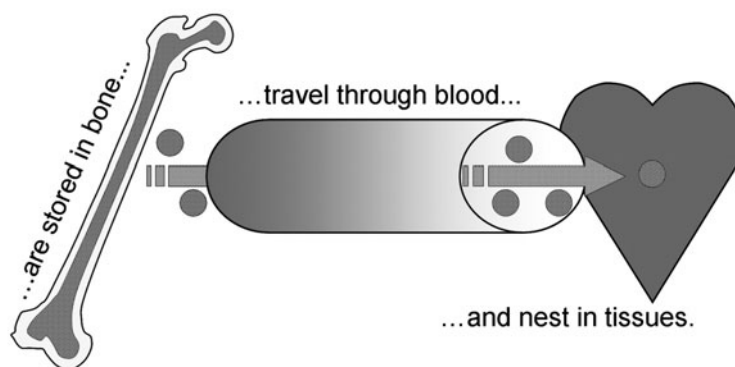
**Figure 3.** Multiplication of human chondrocytes in culture. All the photographs are of the same field, taken at 10 h intervals. The arrows show the cells that are dividing at that moment.

5. **Pancreatic islet transplantation:** Outcomes from this procedure (which is an allogeneic transplant) are, for the moment, worse than those of the combined transplant of kidney and pancreas, which is the procedure of choice, especially if there is diabetic nephropathy. However, application of the Edmonton protocol<sup>15</sup> has notably improved the prospects and there are great expectations with regards to future results. Nevertheless, this procedure should still be considered to be in the experimental phase and needs to be requested as a clinical trial.

## THERAPIES UNDER RESEARCH

The first cells to be used in cell therapy trials were those from the bone marrow. The scientific basis for their use rested on the observation that, in women who had received bone marrow from men there were liver, heart and nervous system cells that expressed the Y chromosome, suggesting that some of the cells of the donor's bone marrow were able transdifferentiate to cells of other tissue types.<sup>8,16,17</sup> Similarly, in male patients who have received a heart transplant from a woman, it is possible to find cardiomyocytes, and endothelial and smooth muscle cells of the intracardiac vessels that do express the Y chromosome. This outcome was unexpected, as all the cells in the transplanted heart should be from the donor (XX) and suggests that cells coming from the host (XY) may colonise the heart and transdifferentiate into cardiac muscle cells and vessels.<sup>16</sup> Accordingly, it was proposed that bone marrow cells, transported in the blood stream, reach peripheral tissues and nest and differentiate into different tissues (Fig. 4). The stimulus that would lead these circulating cells towards the target organ would be the trophic factors released during cell destruction.<sup>18</sup> In an elegant set of experiments, Lin et al<sup>19</sup> demonstrated that, in an animal with ischaemia and reperfusion injury in one of their kidneys, bone marrow cells (identified by genetic markers) nested only in the damaged kidney, where they were transformed into tubular cells, but did not appear in the healthy kidney. Other research supporting this theory included a study in which bone marrow cells (with genetic marker)

Adult stem cells...



**Figure 4.** Bone marrow may work as a reservoir and supplier of progenitor cells, which would have the ability to colonise and proliferate in damaged tissue to regenerate the parenchyma and damaged vessels.



were injected into mice with experimental myocardial infarction. The cells colonised the heart of the host, homing near the infarcted tissue and transforming into cardiac muscle cells and endothelial and vascular smooth muscle cells. Moreover, an improvement in cardiac function was detected in treated mice with respect to the nontreated controls.<sup>20</sup> Since the biosafety with respect to bone marrow cells had been demonstrated in blood treatments, it was possible to pass directly to Phase 1 trials in humans who had suffered from myocardial infarction. Within a few months, several clinical trials were carried out with good results in terms of feasibility and safety and modest results with regards to their effectiveness. To date, this therapy has been applied to more than 1.000 patients. Meta analysis of the results<sup>21,22</sup> shows that in the majority of the trials, the ejection fraction improved between 2 and 10%. In the case of our trial, an in vitro parallel study was carried out on mouse hearts, suggesting that a small percentage of the bone marrow cells (perhaps 1/10.000?) are able to transform into cardiomyocytes.<sup>23</sup> It is doubtful that this low rate of transformation could explain the improvement in the cardiac function. Given this, the current tendency is to attribute a fair proportion of the beneficial effects to the trophic action of the self-transplanted cells.

The bone marrow contains, apart from haematopoietic cells, stromal cells of a mesenchymal lineage, the ability of which to form different tissue types (endothelium, adipose tissue, cartilage, bone, muscle and nervous tissue) is well documented.<sup>10</sup> Furthermore, their biosafety characteristics have been studied in detail.<sup>24</sup> This cell lineage has aroused great interest as a possible candidate for cell therapy. Mesenchymal cells can also be obtained from the adipose tissue, for example, from lipoaspirates in humans,<sup>10</sup> and have been used in several clinical trials, for example, to plug the fistulae that do not heal spontaneously in Crohn's disease, and preliminary results have been satisfactory.<sup>25</sup>

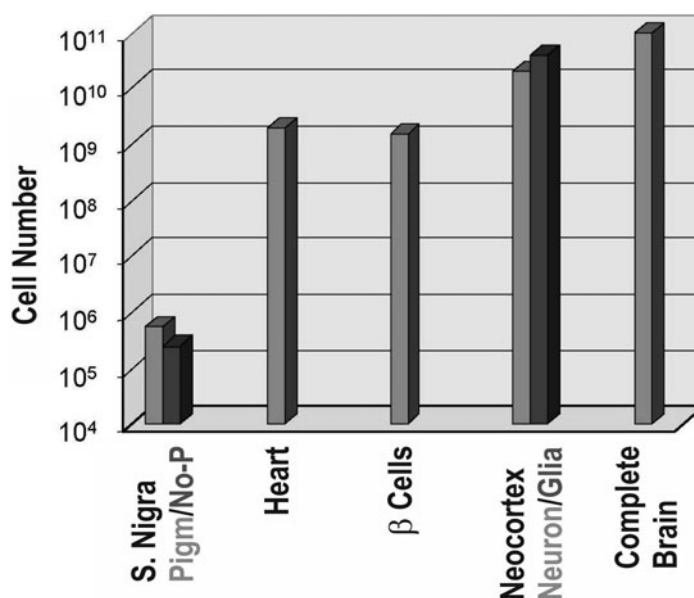
In some therapies, the cells used are not stem cells, or at least they were first used in the belief that they were not. For example, cells from the carotid body, which are very rich in dopamine, have been implanted in the *Substantia Nigra* as a treatment for Parkinson's disease. The outcome has been positive although not spectacular.<sup>26</sup> Surprisingly, recent studies suggest that the carotid body contains a cell kind, Type II cells, that are in fact stem cells and have the ability to produce BDNF (9) which would be the real factor behind the beneficial effects of the implants carried out in the *Substantia Nigra*.<sup>27</sup> Studies with other neural lineages also suggest a very important role of BDNF in the differentiation of nervous system cells.<sup>28</sup>

The following clinical trials are being carried out, within the programme for Advanced Therapies of the Spanish Ministry of Health and Consumption (now the Ministry of Health and Social Policy) in 2008:

- Treatment with skin equivalents for the diabetic foot lesions
- Angiogenesis using mesenchymal cells in peripheral vascular ischaemia
- Treatment of acute and chronic cardiac ischaemia with myoblasts, mesenchymal cells and resident stem cells
- Treatment of amyotrophic lateral sclerosis with bone marrow mononuclear cells
- Treatment of multiple sclerosis with bone marrow mononuclear cells
- Treatment for the nucleus pulposus (intervertebral disc) degeneration with mesenchymal cells
- Mesenchymal cells in spinal arthrodesis
- Expansion of limbal stem cells for the treatment of corneal ulcers

To summarise, the clinical trials currently underway using adult stem cells are mainly focused on cell types the biosafety of which has been well established (bone marrow progenitors and mesenchymal cells) or resident stem cells (limbal stem cells and resident cardiac stem cells). The mechanism of action of the progenitors is not well understood but, even though in many cases there is no sound scientific basis for this, the tendency is to attribute the effects to trophic action.

The number of cells is often cited to support the likelihood of one or other of the mechanisms of action. For example, it is argued that the experiments obtained in hearts of mice cannot be extrapolated to humans, since the number of cells is much higher (about 5,000-fold more) in humans. While this argument has some weight, it remains circumstantial until direct experimental evidence is found. The human adult heart has around  $10^{10}$  cardiomyocytes and a large infarction can destroy about  $3 \times 10^9$  cells. At the high rate of cardiomyocyte production observed after an ischaemic accident ( $5 \times 10^8$ /day), the parenchyma damaged could be regenerated in 1-2 weeks.<sup>29</sup> Could the bone marrow be able to contribute a significant number of cells? This topic has been dealt with in detail elsewhere.<sup>21</sup> We just note here that, in general, 1,000 is taken as the top limit (the “magic number”) for the expansion of progenitors. This limit is equivalent to some 10 consecutive divisions, while a number 10 times greater would correspond to a further 3 divisions. It seems evident that, in terms of probability, 13 divisions are not much less likely than 10. Figure 5 compares the number of cells present in various organs (on a log scale). In the case of the *Substantia Nigra* the figure is less than  $10^6$ ,<sup>6</sup> while in the heart and pancreas it is around  $10^9$  and in the cerebral cortex (a relevant point for Alzheimer’s disease)  $10^{11}$ .<sup>11</sup> Naturally, leaving aside the complexity of the organization of certain tissues, the larger the number of cells destroyed, the more difficult the repair.



**Figure 5.** Number of cells present in various organs. Note the logarithmic scale. In the case of the *Substantia Nigra*, the number represents the pigmented and nonpigmented cells; in the case of the neocortex, neurones and glial cells.

## RESIDENT STEM CELLS

Resident stem cells (RSCs) are very promising but, except for the aforementioned cases listed under Established Therapies, their use is still in the animal experimental phase. In the case of the resident neural stem cells (RNSCs), the main reservoir is in the walls of the cerebral ventricles. It seems that these cells are silent due to the action of local factors, but *in vitro* they form neurospheres which can give rise to both neural and glial lineages. Investigation of the procedures for maintenance and expansion of these neurospheres, as well as the process of differentiation toward specific neuronal types, are very active areas of research.<sup>30</sup>

Reliable observations with regards cardiac resident stem cells (CRSCs) are also very recent.<sup>7,8,30,31</sup> It seems that CRSCs are located in specific niches distributed throughout the heart.<sup>7,31</sup> The expression of stem cells markers has been confirmed in many cases, such as c-kit (stem cell factor receptor), Sca-1 (stem cell antigen 1) and the P-glycoprotein MDR-1, and often all three of them. CRSCs are able to divide asymmetrically, which guarantees their self-renovation. CRSCs would be multipotent stem cells, able to generate precursors for each of the lineages present in the heart: Cardiomyocytes, vascular smooth muscle and endothelium. They can proliferate at a speed of  $5 \times 10^6$  cells per day, but this speed can increase 100-fold or more in certain circumstances (infarction, cardiac hypertrophy induced by aortic stenosis, etc.).<sup>29</sup>

Another possible cardiac progenitor is a curious subpopulation known as cardiac side-population (CSP) cells for their ability to exclude the vital nuclear dye Hoechst 3342. These CSP cells can turn into cardiomyocytes if they interact with adult cardiomyocytes.<sup>32</sup> Side population cells can also be derived from the bone marrow. Another population of progenitors which is present in the adult heart are the cells that express the homeobox islet-1 (*isl-1*) gene. These are present in very small numbers and are restricted to the right side of the heart.

*Isl-1* cells are vestigial primitive cardiomyocytes, but the origin of the other CRSCs is unknown. They may have more than one source, being both remnants of the embryonic period and derived from extrinsic stem cells, circulating in the blood, that nest in the heart (Fig. 4). Indeed, the chimerism found in transplants of bone marrow from males into females (see above) support this hypothesis. The route by which bone marrow cells become CRSCs is unknown; it may occur through transdifferentiation, transdedifferentiation or differentiation of a multipotent precursor present in the bone marrow. In any case, it has been recently demonstrated that some cells of the bone marrow are clonogenic, able to self-renovate and to differentiate into the three cell lineages that are present in the heart.<sup>33</sup> Among bone marrow cell types stromal cells (mesenchymal) seem to be more plastic in terms of potential to differentiate to multiple different lineages. In addition, it has been reported that bone marrow cells may, after an ischaemic lesion, nest in the proximal myocardium and acquire CSP phenotype.<sup>34</sup>

The interstitial space of adult heart contains a large number of cells that do not belong to any of the three previously defined lineages (cardiomyocytes, vascular smooth muscle or endothelial cells) and their function has not been identified. At present there is some evidence that certain of these cells may derive from the epicardium and play the role of CRSCs<sup>35</sup> (Pérez-Pomares, pers. comm.), which would lead to interesting therapeutic possibilities.

## EMBRYONIC CELLS

The use of embryonic stem cells in humans still seems far off, due to the problems mentioned earlier, but basic research with embryonic cells is very active. Two aspects that would bring them closer to clinical practice soon will be mentioned here; nuclear transfer and induced pluripotent stem cells (iPS). Both of these involve the production of embryonic stem cells of the hosts themselves, which would greatly ease the whole procedure and solve all the immunological problems.

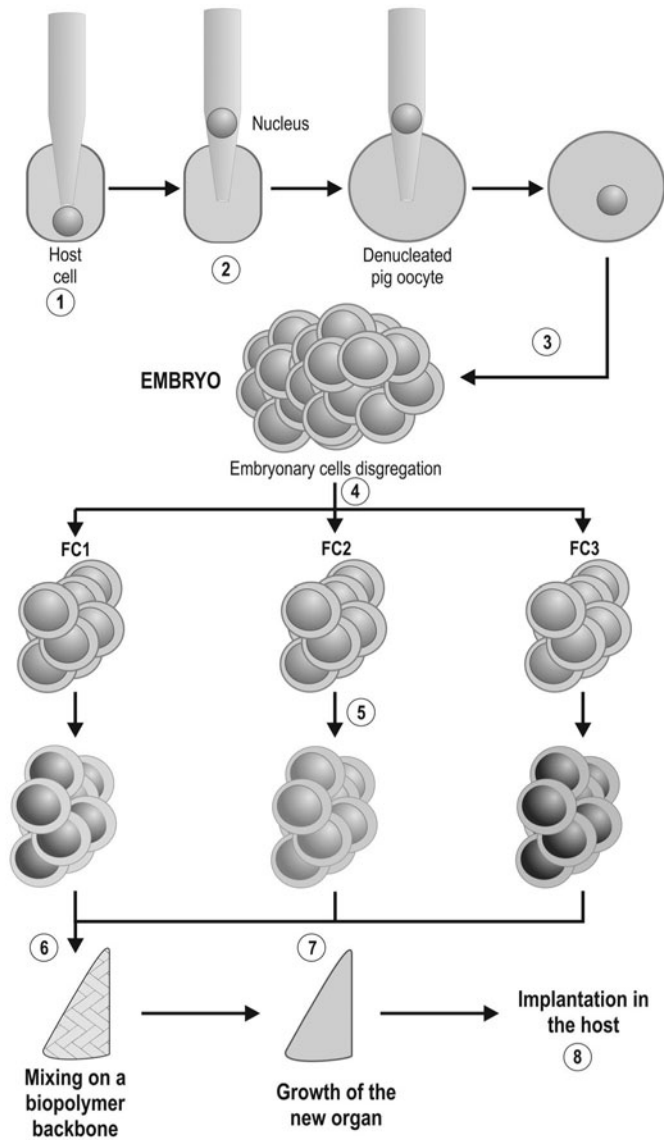
Nuclear transfer consists in transplanting the nucleus of a cell of the host (which contains all its genetic information) into a compatible enucleated oocyte (Fig. 6). Thereby an embryo is created that expresses the genes of the host (stage 3 in Fig. 6); this can serve as the first step towards obtaining the desired differentiated cell types (stages 4 and 5 in Fig. 6), and even to producing engineered organs (stages 6, 7 and 8 in Fig 6). Nuclear transfer has been carried out in animals, but successful transfer of the technique to humans is taking time. In relation to this, the use of nuclear transfer techniques for obtaining material for gene therapy (also called therapeutic cloning) is considered in the Biomedicine Act (see below).

The enthusiasm for the production of engineered organs has declined in recent years, probably because it may be a not very practical solution. The implantation of an artificial organ in the recipient may be very complicated, especially due to nutrition problems. Consider, for example, how could the problem of vascularisation of the graft be addressed when there are no functional vessels in the engineered organ? Spectacular reconstructions have been obtained recently on a matrix obtained from an animal organ.<sup>36</sup> Nevertheless, interest in this field seems to be more academic than practical, since, this type of solution cannot be used in therapeutics for the reasons already discussed.

Not long ago, human iPS cells were generated from skin cells and even more recently this has been achieved from other sources. They were discovered simultaneously by two researchers, Shinya Yamanaka,<sup>37</sup> in Kyoto, and James Thompson,<sup>38</sup> in Wisconsin. The procedure (Fig. 2B), using retroviruses as a tool, involved the over-expression of 4 genes, OCT3/4, SOX2, KLF4 and c-MYC in one case, and OCT3, SOX2, NANOG and LIN28 in the other, in cells taken from the skin. This resulted in “dedifferentiation” of the cells such that they acquire properties similar to embryonic cells and can then differentiate into any type of tissue, that is, they become pluripotent. Since then dedifferentiation has been achieved by manipulating only 2 genes.<sup>39</sup> This type of procedure would enable pluripotent cells to be produced from any cell of the recipients themselves without the need for oocytes or embryos, which make the process much easier, and this has aroused great expectations. The procedure is not yet suitable for clinical use, due to the need to use viruses and to the fact that biosafety issues are not yet resolved (some experimental animals develop teratomas). Alternative procedures to eliminate the requirement for a virus are being designed already, so the production of iPS and nuclear reprogramming may be ready for application at around the same time.<sup>4</sup>

## CONCLUSION

Despite the fact that progress is slow and results are less spectacular than previously envisaged, which tends to be the case with the development of any new treatment, cell



**Figure 6.** Bioengineering of an artificial organ. Growth factors GF1, GF2 and GF3 stimulate pluripotent embryonic cells to differentiate into three different types of tissue, for example, bone, cartilage and skin. Stage 7 also requires growth factors. Alternatively, cell therapy could be carried out by implanting the cells to damaged tissue at stage 6.

therapy has very promising therapeutic potential. This is primarily because it encourages new and imaginative approaches to diseases considered to be incurable, more ambitious end-points and new lines of clinical and basic research. The trials using resident stem cells (chondrocytes, keratinocytes, limbal cells) have had good results, although the scarcity of the starting material may represent a serious limitation. To date, of the

various types of pluripotent stem cells, bone marrow stem cells and mesenchymal stem cells have been the most widely used. The former have the advantage that their biosafety has been demonstrated, while the latter seem to have more plasticity. Bone marrow stromal cells are in between these two types of cells. The therapeutic results of the clinical trials have been modest. So, the time may have come to review the protocols to try to improve the outcomes. In relation to this, it is important to improve our understanding of the mechanisms of action to be able to introduce rational changes to their design. This may require more basic research in association with clinical trials. Research on animal models that are closely related to humans would also be very useful to optimize protocols and demonstrate the biosafety of the new approaches. It should not be forgotten that basic research is the springboard for translation into clinical practice. Embryonic stem cells may require more time become part of the therapeutic arsenal, but this does not mean that they are a less important topic for research. On the contrary, the study of proliferation and differentiation of embryonic cells should be an inspiration for regenerative medicine. Research into different types of stem cells is not counteractive, rather it should be complementary. Studies on nuclear reprogramming and on production of induced pluripotent cells are very promising fields, that unite the use of embryonic and adult stem cells.

## ACKNOWLEDGMENTS

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## CHAPTER 8

# CHARACTERISTICS OF ADULT STEM CELLS

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**Abstract:** Stem cells are characterized by their unlimited ability to divide specifically; a stem cell is capable of making an immense number of copies of itself, maintaining the same characteristics. Moreover, these cells are able to generate several of the cell lineages which make up the body, including cells from the heart, liver, kidney, neurons, and muscles. Investigation of the mechanisms through which this differentiation occurs, the genes involved and the possibility of increasing the efficiency with which stem cells can be isolated and/or characterized are currently among the most important fields in biology and biomedicine.

To date, stem cells have been identified from four different sources: Embryonic stem cells (ESC), germinal stem cells, and those derived from embryonic carcinomas (teratocarcinomas) and from somatic tissues (somatic stem cells). The latter are called adult stem cells (ASC) when they are found in postnatal tissues. We now know that there is a great diversity among ASC, with some tissues, such as the bone marrow, containing more than one type of ASC. Adult stem cells have several characteristics that make them to be the main players in current regenerative medicine and are being investigated as potential therapeutic agents for a wide variety of diseases. Specifically, HSC and MSC are being assessed in increasing numbers of clinical trials.

## INTRODUCTION

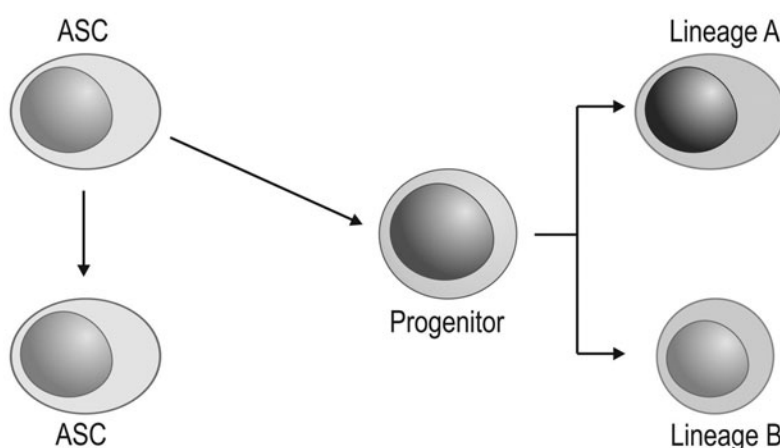
There are two main characteristics that define stem cells (SC) and that make them different from the great majority of cells that constitute the adult body. First, in suitable conditions, they have an unlimited ability to divide; specifically, a stem cell is capable of making an immense number of copies of itself, maintaining the same characteristics. By contrast, other somatic cells, from which we are all constituted, are limited to a fixed

number of divisions (it is estimated that they can divide around fifty times after which they age and eventually die). Second, SC are able to generate several of the cell lineages which make up the body, including cells from the heart, liver, kidney, neurons, and muscles. To date, stem cells have been identified from four different sources: Embryonic stem cells (ESC), germinal stem cells, and those derived from embryonic carcinomas (teratocarcinomas) and from somatic tissues (somatic stem cells). The latter are called adult stem cells (ASC) when they are found in postnatal tissues.

Until a decade ago, the only mammal ASC that had been conclusively proved to exist were hematopoietic stem cells (HSC), which are responsible for the production of all blood cell lineages. The existence of ASC in other highly proliferative tissues such as skin and liver had been proposed, but the generic concept of “adult stem cell” as a type of cell occurring in all tissues is quite recent. We now also know that there is greater diversity among ASC, with some tissues, such as the bone marrow, containing more than one type of ASC (Table 1).

### DIFFERENTIAL CHARACTERISTICS OF ADULT STEM CELLS

During normal cell division, the two daughter cells produced are equivalent and the same as the mother cell from which they are derived (symmetric division). After this, the descendant cells can evolve along different routes, either following certain programmes of differentiation or retaining the potential of their initial state. Maintaining a suitable balance between the rates of proliferation and differentiation enables most tissues to establish homeostatic control of their shape and size, avoiding uncontrolled growth, associated with, for example, tumour formation. On the other hand, SC seem to be regulated by a conservative division mechanism (asymmetric), in such way that the division produces one cell identical to the mother cell and the other is in charge of the rest of the differentiation programme. In theory, this mechanism should allow strict regulation of the number of SC existing in any given organ (Fig. 1).



**Figure 1.** Asymmetric division in stem cells. An adult stem cell (ASC) can divide to generate another ASC and a cell compromised to differentiate into specific cell lineages (progenitor).

It has been assumed that SC present in the adult body (ASC) must have, in general terms, less potential than those present during the development of the embryo and foetus. However, although this is easy to assume in principle, it is much harder to demonstrate. Further, at least in the adult body, not all the stem cells of an organ actively participate in the processes of regeneration and maintenance of the functionality. At a given time, only a certain proportion of cells are simultaneously contributing to the production of new tissue. The rest remain in a resting state (known as quiescence), being protected from external attack, physical or chemical, as well as from the process of cell ageing. When the SC in charge of tissue regeneration becomes worn out, the progeny of others progressively substitutes them. The new cells produced in this way are different clones, and the phenomenon responsible for the process is known as “clonal succession”.

In short, a SC is functionally defined as a cell that is capable of self-maintenance (through the process of asymmetric division) and has the potential to generate (through the differentiation of daughter cells) various or all cell lineages (multipotency or pluripotency respectively). Therefore, not all stem cells are identical, and although most are defined by their origin (embryonic, adult, etc.), these terms may not necessarily reflect a greater potential for development or, even, in terms of potential biomedical applications. Investigation of the mechanisms through which this differentiation occurs, the genes involved and the possibility of increasing the efficiency with which stem cells can be isolated and/or characterised are currently among the most important fields in biology and biomedicine.

## TYPES AND SOURCES OF ADULT STEM CELLS

The embryonic origin of all types of ASC is, ultimately, the same as other somatic cells present in the adult body, that is, the pluripotent cells of the inner cell mass of the blastocyst. However, little is known about the specific precursors of each type of ASC, beyond the germ layer to which they belong (ectoderm, mesoderm or endoderm).

As with other aspects of their biology, the embryonic source of HSC is the best known of all types. Since the early 20th century, it has been believed that HSC derive from a more primitive ancestor, common to the endothelial lineage, known as hemangioblast. The existence of hemangioblasts has been confirmed by several experimental studies in various stages of completion.<sup>1</sup> Hematopoiesis starts in the embryo of mammals in the vitelline sac (fifth week of gestation in humans; 7-7.5 days in mice). However, the hematopoietic precursors of the vitelline sac are only responsible for the embryo hematopoiesis, and do not give rise to the HSC found in the adult. In fact, the capacity of primitive progenitor cells (CD34<sup>+</sup>/CD117<sup>+</sup>) isolated from the vitelline sac to contribute to hematopoiesis in adults is limited. The HSC responsible for the definitive hematopoiesis in adults come from the mesoderm region known as aorta-gonad-mesonephros (AGM, derived from the para-aortic splanchnopleura). After the vitelline sac, hematopoiesis takes place in the spleen, liver and lymph nodes, until the bone marrow, which eventually takes charge of producing blood cells for whole adult body, develops.

Bone marrow stem cells represent approximately 1 per 10.000 mononuclear cells; they also can be obtained from peripheral blood, since they are able to leave the bone marrow and go into the blood stream, in a process known as mobilisation. ASC are characterised by their small size, high nucleus-cytoplasm ratio, poor staining ability with vital dyes such as Hoechst 33342, absence of lineage markers (Lin-) and presence of various surface

**Table 1.** Major types of adult stem cells (ASC) described in mammals. The in vivo occurrence of some of them, as well as their actual differentiation potential in physiological conditions, are still controversial. The cell markers are in most cases only proposals, and none of them allows the unequivocal identification of a SSC. CNS, Central Nervous System; PNS, Peripheral Nervous System; SVZ, Subventricular Zone. Note from the authors: Not all the references in this table have been included in the References section, due to space constraints.

Type	Localization	Markers		Potential	References
Hematopoietic (HSC)	- Bone marrow	Mouse:	Human:	- All hematopoietic lineages	Osawa et al, 1996
	- Blood	CD34 <sup>+</sup>	CD34 <sup>+</sup>	- Liver	Lagasse et al, 2000
	- Other tissues?	CD117 <sup>+</sup>	CD38 <sup>-</sup>	- Muscle	Krause et al, 2001
Mesenchymal (MSC)		Sca-1 <sup>+</sup>	CD59 <sup>+</sup>	- Epithelial tissues	Kiel et al, 2005
		Lin <sup>-</sup>	CD90 <sup>+</sup>		
		CD150 <sup>+</sup>	CD117 <sup>+</sup>		
		CD48 <sup>-</sup>	Lin <sup>-</sup>		
	- Bone marrow	CD44 <sup>+</sup>		- Bone	Pittenger et al, 1999
	- Adipose tissue	CD45 <sup>-</sup>		- Adipose tissue	Zuk et al, 2002
	- Cartilage	CD34 <sup>-</sup>		- Cartilage	De la Fuente et al, 2004
	- Bone	CD105 <sup>+</sup>		- Tendons	
	- Skeletal muscle			- Skeletal muscle	
	- Blood			- Smooth muscle	
Neural (NSC)	- Other tissues?			- Cardiac muscle	
				- Endothelium	
				- Neurons	
				- Astrocytes	
	- CNS:			- Neurons (all types)	Doetsch et al, 1999
	- SVZ, Hippocampus, Olfactory bulb	Nestin <sup>+</sup>		- Glia	Clarke et al, 2000
				- All somatic lineages?	
	- SNP:	p75 <sup>+</sup>		- Neurons	Kruger et al, 2002
	- Gut	Integrin $\alpha 4^{+}$		- Glia	
				- Smooth muscle	

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Table 1. Continued

Type	Localization	Markers	Potential	References
Epidermic	- Basal layer of interfollicular epidermis	p63 <sup>+</sup> Keratin 15 <sup>+</sup> Integrin $\alpha 6^{+}$ CD71 <sup>-</sup>	- Epidermis	Pellegrini et al, 2001 Webb et al, 2004
		Mouse: CD34 <sup>+</sup> Keratin15 <sup>+</sup> Integrin $\alpha 6^{+}$		
Endothelial	- Hair follicle (bulge)	Human: CD34 <sup>-</sup> CD24 <sup>-</sup> CD71 <sup>-</sup> CD200 <sup>+</sup> CD146 <sup>-</sup>	- Epidermis and apandages (Hair follicle and sebaceous gland) - Neuronal - Mesoderm	Tumbar et al, 2004 Blanpain et al, 2004 Morris et al, 2004 Ohyana et al, 2006
		Sca-1 <sup>+</sup> (mouse) CD34 <sup>+</sup>		
		CD117 <sup>+</sup> CD133 <sup>+</sup> KDR <sup>+</sup> Tie-2 <sup>+</sup>		
		- Bone marrow - Blood		Asahara et al, 1997 Shi et al, 1998 Harraz et al, 2001
Pancreatic	- Pancreatic ducts and islets	PDX-1 <sup>+</sup> Ngn3 <sup>+</sup>	- Endocrine pancreas ( $\alpha$ , $\beta$ , $\delta$ cells) - Exocrine pancreas - Stellate cells - Neurone - Glia	Seaberg et al, 2005 Xu et al, 2008
Muscle (Satellite cell)	- Scheletal muscle	Pax7 <sup>+</sup> Sca-1 <sup>-</sup> (mouse) CD34 <sup>+</sup> CD45 <sup>-</sup>	- Myocytes - Hematopoietic cells	Jackson et al, 1999 Howell et al, 2002 Montarras et al, 2005

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Table 1. Continued

Type	Localization	Markers	Potential	References
Cardiac (CSC)	- Myocardium	CD117 <sup>+</sup> / Sca-1 <sup>+</sup> (mouse) CD45 <sup>-</sup> KDR <sup>+</sup>	- Cardiomyocytes - Smooth muscle - Endothelium - Neurons - Glia - Bone - Fat	Beltrami et al, 2003 Oh et al, 2003 Messina et al, 2004 Pfister et al, 2005 Barile et al, 2006
Hepatic (Oval cell)	- Liver	Cytokeratins 8/18 <sup>+</sup>	- Hepatocytes - Biliary duct epithelium	Thorgeisson, 1993 Dabeva et al, 1993 Allain et al, 2001
Renal	- Kidney	CD133 <sup>+</sup> Sca-1 <sup>+</sup> (mouse) Oct4 <sup>+</sup> Pax-2 <sup>+</sup>	- Renal tubules - Neurons - Bone - Fat - Liver - Endothelium	Oliver et al, 2002 Maeshima et al, 2003 Iwatani et al, 2004 Kitamura et al, 2005 Challen et al, 2006 Gupta et al, 2006
Intestinal	- Crypts of the intestinal epithelium	Musashi-1 <sup>+</sup> Hes-1 <sup>+</sup>	- Intestinal epithelium (Enterocytes, Goblet cells, Paneth cells, and Enteroendocrine cells)	Potten et al, 2002 Kayahara et al, 2005

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Table 1. Continued

Type	Localization	Markers	Potential	References
Corneal	- Basal layer of the corneal limbus	Keratin 3/12 <sup>-</sup> Keratin 5/14 <sup>+</sup> p63 <sup>+</sup> ABCG2 <sup>+</sup>	- Corneal epithelium	Cotsarelis et al, 1989 Budak et al, 2005
Retinal	- Retinal cilliary margin	Pax6 <sup>+</sup>	- Retina (Photoreceptors, bipolar neurons, Müller glia)	Tropepe et al, 2000 Coles et al, 2004
Dental	- Dental pulp	STRO-1 <sup>+</sup> Nestin <sup>+</sup> CD146 <sup>+</sup>	- Odontoblasts - Adipocytes - Neurons	Gronthos et al, 2000 Miura et al, 2002
MAPC	- All tissues?	CD44 <sup>-</sup> HLA-1 <sup>-</sup>	- All somatic linages?	Reyes et al, 2001 Jiang et al, 2002



markers such as: CD34, CD90, CD117 (c-kit) and CD133, among others. It is possible to obtain a population highly enriched with HSC using flow cytometry to select cells that express any of the aforementioned markers (traditionally CD34). However, none of these markers alone or in simple combinations enable the specific identification of an HSC.

Apart from HSC, the bone marrow has at least one other type of ASC known as mesenchymal stem cells (MSC), which are fibroblastoid cells precursor of all nonhematopoietic connective tissues (bone, adipose tissue, cartilage, etc.).<sup>2</sup> These cells are not only found in the bone marrow, but also in the stroma of virtually all tissues, for example the subcutaneous adipose tissue<sup>3</sup> and articular cartilage.<sup>4</sup> MSC are mainly obtained through selection using plastic adhesion in cell cultures, since they are able to adhere and grow in conditions in which other cell types do not normally proliferate. They do not have any specific markers, but have a homogenous and reproducible profile of surface antigens including: CD9<sup>+</sup>/CD13<sup>+</sup>/CD29<sup>+</sup>/CD14<sup>-</sup>/CD34<sup>-</sup>/CD44<sup>+</sup>/CD45<sup>-</sup>/CD90<sup>+</sup> and CD105<sup>+</sup>.

Thanks to the fact that they are easy to obtain, their high *ex vivo* proliferation capability (in contrast to HSC) and their high differentiation potential, MSC are one of the types of ASC most used in cell therapy. Also, under some experimental conditions, MSC have demonstrated the ability to differentiate to nonconnective cell lineages, such as endothelial and neuronal lineages. Finally a particularly interesting property of MSC is that they are able, both *in vitro* and *in vivo*, to inhibit the immune response.<sup>5</sup> This ability to immunoregulate includes inhibition of the activation of T, B and NK cells and of the maturation of dendritic cells, as well as protection against inflammatory and/or autoimmune disorders, including transplant rejection.

Outside the bone marrow, one of the tissues where it has been assumed for decades that there were stem cells, given its highly proliferative nature, was the epidermis. To date, the existence of epidermal stem cells (EpSC) has been confirmed in at least two different locations. First, it is estimated that between 1 and 2% the interfollicular basal layers is EpSC, with p63<sup>+</sup> phenotype, which are able to produce keratinocytes that, migrating to the surface, form the epidermis.<sup>6</sup> Secondly, a more primitive stem cell population, CD34<sup>+</sup>, Keratine 15<sup>+</sup> and Integrin  $\alpha 6$ <sup>+</sup> phenotype (in mice), have been found in the interior of hair follicles, specifically in the bulge region. These cells are not only able to form the epidermis itself, but also to the follicles and sebaceous glands. However in humans, the identification of interfollicular EpSC has proved more difficult, and several different phenotypes have been proposed for bulge EpSC, one of them being CD200<sup>+</sup>/CD34<sup>-</sup>/CD24<sup>-</sup>/CD71<sup>-</sup>/CD146<sup>-</sup>.<sup>7</sup> As with other types of ASC, there are studies indicating that EpSC show plasticity under certain experimental conditions.<sup>8</sup>

Unlike the bone marrow or the epidermis, both tissues with a high cell turnover rate, the discovery of SC in the CNS was, without any doubt, one of the most striking findings at the beginning of this century.<sup>9</sup> Neural Stem Cells (NSC), present in the adult brain of mammals, originates from the cells of the neural crest and they are mainly located in the subventricular zone and subgranular zone in the dentate gyrus of the hippocampus. The main marker of these cells is nestin and they are isolated using cell culture in the presence of EGF and bFGF growth factors, growing in free floating spherical aggregates, known as neurospheres. Neurosphere culture makes possible to obtain large quantities of NSC, which are then able to differentiate both to neurons and glia (astrocytes and oligodendrocytes).

The hematopoietic, mesenchymal, neural and epidermal SC are the most studied and best understood types of SC so far, but as shown in Table 1, they are not the only type of

SC that have been demonstrated to be in adult somatic tissues. Other types of ASC that have attracted special attention in regenerative medicine include:

- Endothelial stem cells. Endothelial precursors have been described (that some authors consider to be stem cells) both in the bone marrow and circulating.<sup>10</sup> These cells are linked to hemangioblasts, and have aroused great interest due to their potential use in the treatment of ischemic pathologies.
- Skeletal muscle stem cells. Traditionally referred to as satellite cells, these SC are defined by the expression of the transcription factor Pax3. In mice, it has been demonstrated that this population has the phenotype CD34<sup>+</sup>/CD45<sup>-</sup>/Sca1<sup>-</sup>.<sup>11</sup>
- Pancreatic stem cells. Experimental evidence suggests there are one or more types of stem cells in the pancreas. Stem cells able to differentiate to beta cells seem to be present in the islets and in the pancreatic ducts, which are neurogenin-3 positive.<sup>12</sup>
- Cardiac stem cells. Several groups have reported in recent years, successful isolation of cells able to differentiate to cardiomyocytes, endothelium and smooth muscle from myocardium, although there is no consensus over their importance, origin and phenotype. Most studies identify as cardiac stem cells those that are CD117<sup>+</sup>/Sca1<sup>+</sup>.<sup>13</sup>

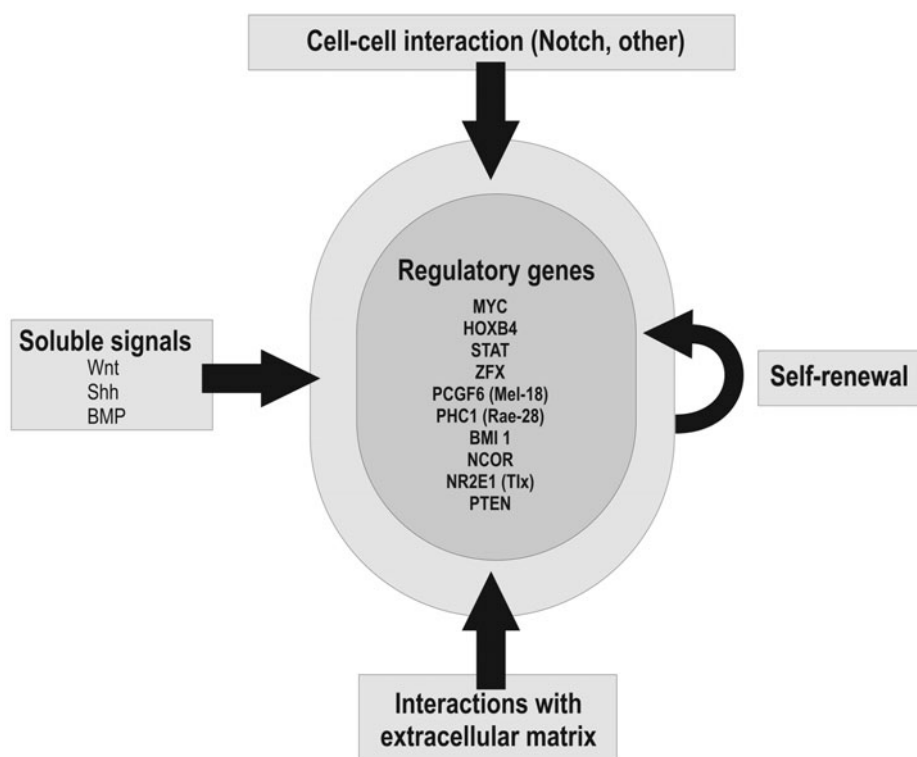
Finally, several studies have described the existence of ASC that are pluripotent (or at least, that have great multipotency) in mammal tissues. The work of Dr. Catherine Verfaillie stands out: With her group reporting successful isolation, from bone marrow and other tissues of several mammal species, of cells that are able to differentiate both in vitro and in vivo to almost all the somatic cell lineages. They have termed these cells MAPC (Multipotent Adult Progenitor Cells) and they are similar to MSC but negative to the markers CD44 and HLA-I.<sup>14</sup> However, many groups have failed to satisfactorily reproduce these finding, and currently MAPC are generally considered to be the result of some kind of modification induced by the ex vivo culture conditions rather than a type of cell that exists in in vivo physiological conditions.

## THE CONCEPT OF NICHE AND THE CONTROL OF SELF-RENEWAL

Stem cells are, together with tumour cells, the only present mammalian cells that are able to proliferate and maintain their differentiation potential indefinitely, in some cases (as for HSC) for the entire life of the individual. This ability to divide limitlessly to give rise to other identical/equivalent SC is what is known as self-maintenance or self-renewal.

Self-renewal is a biological process of great importance, since it holds the key to tissue regeneration and homeostasis, as well as growth, ageing and cancer. Despite this, our understanding of this process is still very limited. Most of what we now know about the mechanisms of self-renewal has been learned from the ESC model. In this model, a series of extracellular signals (LIF, BMP, FGF, etc.) have been identified that contribute to maintaining the expression of various transcription factors (Oct4, Nanog and Sox2, among others), which themselves work as master genes that maintain the pluripotent state of cells.<sup>15</sup> Recently, several combinations of such genes have been identified, the exogenous expression of which, in differentiated cells, can by itself make such cells pluripotent.<sup>16</sup>

The potential role of the aforementioned pluripotent genes in ASC self-renewal is not well understood, but all the evidence points to their function being quite specific to ESC, and that in ASC self-renewal is mainly regulated by other combination of factors. On the



**Figure 2.** Some extracellular signals and genes involved in regulation of ASC self-renewal.

other hand, a feature shared by the self-renewal mechanisms of all SC is a dependence on a specific set of extracellular signals, which is different for each type of SC and includes: Soluble paracrine and autocrine signals, interactions with the extracellular matrix, and cell-cell interactions (Fig. 2). According to their effect, such self-renewal signals for cell maintenance can be classified as:

- Proliferation signals: These induce cell division.
- Quiescence signals: These induce the slowing down of the cell cycle such that the cells enter the so-called G0 phase, a state they can stay in for long periods of time without differentiating or suffering genetic damage.
- Differentiation inhibitory signals: These contribute to maintaining the expression of genes that inhibit cell differentiation processes.
- Survival signals: These provide a signal without which the SC would go into senescence/apoptosis or protection from the action of other signals able to induce said apoptosis.

This set of signals, which together constitute the specialised microenvironment of a given SC, is what is called niche. The concept of niche, derived, once again, from the study of the hematopoietic system, is essential for the understanding of the biology of the SC. In a suitable niche, SC are able to self-renew; outside the niche, SC differentiate or die. Each type of ASC therefore lives only in very specific anatomical locations within the

body, places where the signals constituting the corresponding niche are provided. This strict control of self-renewal is one of the main differences between stem and tumour cells, the latter not seeming to be under such restrictions.

The niches of some types of ASC have been located accurately, for example the aforementioned EpSC (in the epidermal basal layer and follicular bulges), the NSC (in the subventricular zone and dentate gyrus) and the intestinal SC, found in the crypt base. On the other hand, in other types of ASC, the location of the niche still remains unclear. In the case of HSC, they have been reported to be mainly located near the surface of the bone, but also associated to sinusoidal endothelium.<sup>17</sup> So far, neither the relationship between these two types of niche nor their likely different functions in the biology of HSC are totally understood.

The main regulatory signalling pathways for ASC are Wnt, Notch and Hedgehog.<sup>18</sup> A common characteristic of all of these signals is that they can regulate self-renewal in various ways (inducing or inhibiting it), depending on the context, and probably as a result of quantitative equilibria. For example, activation of the Notch signalling pathway is essential for self-renewal of HSC, but it can also promote self-renewal of NSC in some cases as well as glial differentiation in others.

Wnt signalling through the canonical route ( $\beta$ -catenin) promotes self-renewal in HSC, MSC, NSC, EpSC and intestinal SC. However in HSC, the deletion of  $\beta$ -catenin does not affect the capacity for self-renewal, which suggests that either Wnt signalling is not necessary or it works through the noncanonical pathway in these cells. In contrast, the accumulation of Wnt-3a in the serum of mice has recently been associated with the phenomenon of physiological ageing, at least for muscle satellite cells.<sup>19</sup>

*Sonic Hedgehog* (Shh) also promotes the maintenance of HSC, NSC and EpSC. At least in HSC, said activity takes place through a mechanism dependant on BMP-4. The signalling route of TGF- $\beta$ /BMP (mediated by Smads) has been extensively studied in HSC, and it is known that TGF- $\beta$  is one of the most potent HSC self-renewal inhibitors. Due to the high level of redundancy among Smad proteins, their role in the self-maintenance of ASC is still not well understood.

The specific genetic programmes in charge the control of self-renewal of ASC are considerably less well identified than the extracellular signals that regulate them. Some of these are listed in Table 2. To date, the most studied self-renewal genes in ASC are the Polycomb family of proteins. Polycomb-group proteins (PcG) are associated with two large complexes that suppress the transcription of other genes by modifying the structure of chromatin. Some PcG genes have been found to participate in the regulation of ASC self-renewal. Among these, the best known is Bmi-1,<sup>20</sup> the expression of which

**Table 2.** Main known genes involved in self-renewal regulation in ASC

Functions	Genes
Transcription factors	MYC, SOX2/10, HOXB4, ZFX, PAX6/7
Signalling pathways	Wnt, Notch and Shh pathways; SMADs; STATs
Polycomb group	BMI1, PCGF6, PHC1
Nuclear receptors	NCOR1, NR2E1
Cell cycle regulators	CDKN2C, CDKN1A, PTEN

is necessary for the maintenance of EpSC and NSC. Bmi-1 promotes the proliferation of ASC, mainly by suppressing the cellular senescence pathway induced by p16<sup>Ink4a</sup> and p19<sup>Arf</sup>.

MAIN CURRENT APPLICATIONS

Adult stem cells have several characteristics that make them to be the main players in current regenerative medicine. First, their natural function is the maintenance/repairing of adult body tissues, so they have potential direct clinical applications. Secondly, autologous forms can be obtained in the majority of cases, without great difficulty and with minimal manipulation. Finally, their biological potential is greater than previously thought, since they can be considerably expanded ex vivo and have a broad differentiation potential. For all these reasons, ASC are being investigated as potential therapeutic agents for a wide variety of diseases. Specifically, HSC and MSC are being assessed in increasing numbers of clinical trials (Table 3).

In fact, some of the transplant strategies used for several decades, such as artificial skin grafts, pancreatic islets transplantation and articular cartilage repair, could be considered as applications of ASC, since their therapeutic effect is ultimately mediated by populations of these cells. In the current chapter, however, we only refer to the applications of well characterized, isolated ASC populations with homogenous phenotypes.

**Table 3.** Some examples of clinical trials in cell therapy using HSC and MSC (Source: Clinicaltrials.gov)

ASC Type	Pathology	Reference
HSC	Peripheral ischemia	NCT00616980
	Myocardial infarct	NCT00313339
	Chronic wounds	NCT00535548
	Multiple sclerosis	NCT00273364
	Cirrhosis	NCT00713934
	Miastenia gravis	NCT00424489
	Tibial fracture	NCT00632034
MSC	Peripheral ischemia	NCT00518401
	Myocardial infarct	NCT00114452
	Tibial fracture	NCT00250302
	Cirrhosis	NCT00420134
	Perianal fistula	NCT00475410
	Crohn disease	NCT00482092
	Transplant rejection	NCT00658073
	Multiple sclerosis	NCT00395200
	Graft-versus-host disease	NCT00136903
	Lupus erythematosus	NCT00698191

Broadly speaking, we can group the therapeutic applications of ASC that are currently being studied or in clinical use into the following classes:

- Use of ASC to directly repair tissue damage (through mechanisms of cell proliferation/differentiation), e.g., bone marrow transplantation (HSC) after myeloablation induced by chemo/radiotherapy.
- Use of ASC as ex vivo gene therapy vectors, e.g., transplantation of genetically modified autologous bone marrow to repair an immunodeficiency. The transplant of allogeneic ASC to correct genetic defects may also be included in this category.
- Use of ASC to promote angiogenesis in ischemic areas, e.g., transplantation of HSC into patient suffering from peripheral ischemia.
- Use of ASC as inhibitors of inflammatory/immune processes, e.g., transplant of MSC for the treatment of Graft-versus-host disease (GVHD).

The great majority of clinical procedures using ASC are based on HSC. Bone marrow transplantation (transplant of populations of HSC) has been carried out fairly routinely across the world for decades, for the treatment of many diseases, including leukaemia, lymphoma, myeloma, myelodysplastic syndrome, aplastic anaemia and immunodeficiencies, among others. Transplantation of autologous HSC that have been genetically modified has also been used experimentally in clinical trials for more than a decade to correct monogenic diseases of the lymphohematopoietic system, such as severe combined immunodeficiency.<sup>21</sup>

Second to HSC, the ASC most widely used in studies have been MSC. In fact, currently, MSC can be considered to be the most promising type of ASC for treating a great variety of degenerative diseases. Among these, the following diseases stand out for the clinical development:

- Diseases and lesions of connective tissues. As cellular precursors of nonhematopoietic connective tissues, MSC are the logical candidates to contribute to the repair of lesions in these tissues. Given this, several studies have tackled the challenge achieving repair of bone, cartilage, tendon and muscle using cell therapy using MSC.<sup>22</sup> Specifically in the case of cartilage, autologous chondrocyte implantation can be considered as an application of ASC, since it has been demonstrated that the cells used, which have been known as “dedifferentiated chondrocytes”, are in fact MSC.<sup>4</sup> Similar to the use of HSC for repairing genetic defects in the hematopoietic system, MSC have been proposed as optimal agents to repair genetic diseases affecting bones. Specifically, the transplantation of allogeneic or autologous MSC stem cells that have been genetically modified is being investigated for the treatment of osteogenesis imperfecta.<sup>23</sup>
- Myocardial infarction. Like HSC, MSC are also being investigated as a potential treatment for alleviating the loss of cardiac function after myocardial infarction. Results seem to demonstrate higher efficacy of the MSC, but the specific mechanism involved remains unknown, and, as occurs with HSC, there is no long-term significant graft of the transplanted cells.<sup>24</sup>
- Digestive system fistulas. Implantation of MSC derived from adipose tissue is being studied as a treatment of complex perianal fistulas. Results of the clinical study carried out are promising,<sup>25</sup> suggesting that this cell therapy may be a valid alternative to surgery for the repair of this type of lesion.

Another use of MSC, as immunoregulatory agents, deserves a mention. The ability of MSC to inhibit the immune response has opened a new field in cell therapy, focused on the



treatment of diseases with an inflammatory or autoimmune component. The biological activity of MSC is being investigated both at preclinical and clinical levels in cell therapy studies for various diseases, such as rejection of allogeneic, graft-versus-host disease, experimental autoimmune encephalitis, Crohn's disease and collagen-induced arthritis.<sup>5</sup> Moreover, this immunosuppressive activity goes hand-in-hand with the hypo-immunogenic characteristic, that enables MSC to escape from alloreactive recognition, and this suggests the wider potential of allogeneic MSC for generalised use in clinical applications.

There are other type of ASC that are being investigated as potential therapeutic agents in various diseases, although currently their use is much more restricted than of HSC and MSC. Specifically, for example, NSC are being studied as a potential treatment for spinal cord injuries, Parkinson's disease and other degenerative pathologies of the central nervous system.<sup>26</sup> However, to date the great difficulty of obtaining NSC from adult donors has limited their clinical use.

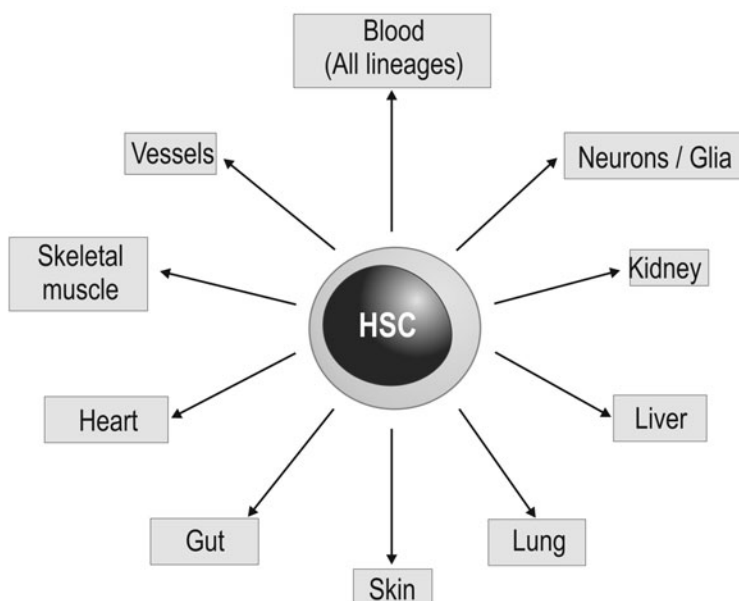
## PLASTICITY OF ADULT STEM CELLS

In the last ten years, there has been controversy, and in many senses there still is, over the novel concept of cell "plasticity" which is mainly based on the results of experiments using HSC (Fig. 3). Cell plasticity is the phenomenon by which an ASC, extracted from its natural niche, expanded *ex vivo* or not, and transplanted to another physiological environment is able to produce cell lineages other than those expected under its normal programme of development. Though it is far from clear, various mechanisms have been implicated in this process, including dedifferentiation, transdifferentiation, and reprogramming. Some of the initial postulates have not been confirmed but others authors.<sup>27</sup> Currently cell therapy using HSC is being investigated as a therapeutic procedure for the treatment of many pathologies not related to the lymphohematopoietic system. In particular, the main potential applications are liver repair/regeneration and the treatment of ischemia.

The ability of HSC to produce new liver cells has been demonstrated in various animal models of liver insufficiency,<sup>28</sup> and their potential clinical use is very promising for the treatment of these pathologies. However, it has been confirmed that the production of new hepatocytes is not due to the differentiation of HSC, but rather to a process of fusion of these cells (or other derived from them, such as macrophages) with pre-existing hepatocytes.<sup>29</sup> Nevertheless, this finding does not completely invalidate the concept of treating liver diseases through HSC transplantation, and the biological properties of the cells derived from these fusions are currently being investigated. Similarly, it has very recently been confirmed that lymphoid and myeloid cells can merge, *in vivo*, with muscle cells, hepatocytes, cardiomyocytes and Purkinje neurons, although this mechanism seems to be relevant only in cases of severe damage and inflammation.<sup>30</sup>

Another disease that is being addressed with the use of HSC transplantation is cardiac ischemia. Initial studies in rodents<sup>31</sup> encouraged the development of many preclinical and clinical cell therapy studies driven by the hypothesis that HSC had sufficient plasticity for transdifferentiation to heart and muscle cells lineages. However subsequent studies have seemed to rule out a significant proportion of transdifferentiation taking place under normal experimental conditions,<sup>32</sup> although *in vivo* transdifferentiation has been observed in various studies carried out in men that received a heart transplant from a female donor, in which there was no apparent mediation of fusion mechanisms.<sup>33</sup> Consequently, the





**Figure 3.** Plasticity of hematopoietic stem cells. Figure shows cell lineages derived from HSC in several in vivo models. In many cases it has been demonstrated that these lineages have been originated by cell fusion between the HSC and differentiated cells of the corresponding non-hematopoietic tissues.

controversy is ongoing. On the other hand, the potential proangiogenic ability of HSC is being actively investigated for the treatment of peripheral ischemia.<sup>34</sup>

Similarly, the potential of HSC for in vivo transdifferentiation to central nervous system cells has also been validated in various studies carried out on women who had received a bone marrow transplant from a male donor.<sup>35</sup>

In short, the cell plasticity of the different ASC is a new therapeutic possibility that will need much more time to be properly evaluated, and which should not be ruled out for the treatment of certain conditions.

## CONCLUSION

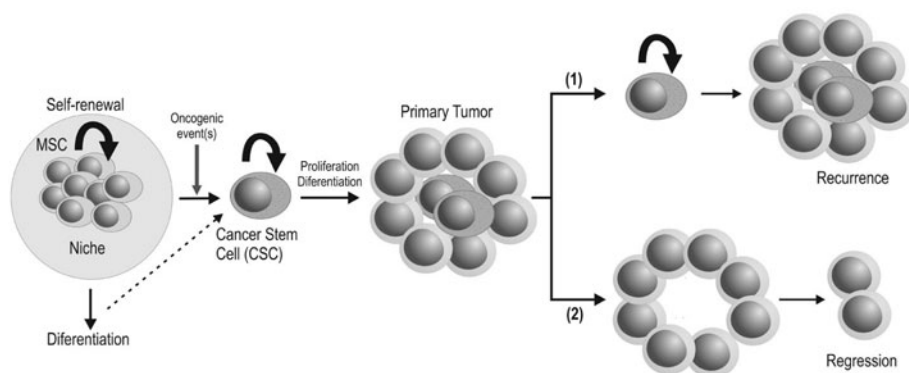
We should continue to encourage the interest and social support that the potential of stem cell therapy has aroused, but at the same time must maintain high standards and extreme caution. It is important to communicate information to the wider society in a balanced way, explaining that any significant progress in the laboratory will take more than 10 years to become an established clinical reality.

The expectations raised when it first emerged that there was a possibility of introducing into the living body new “correct” versions of the genes that had mutations, associated to human genetic diseases (gene therapy), were extraordinary. Despite the best efforts, the reality has been much more difficult than imagined, and we have had to wait more than 25 years to see the first clinical benefits (and the treatments are still not trouble free).

We all, researchers, clinicians and patients, have felt the consequences of this initial over-optimism. We sincerely hope that the same will not occur in the case of the infinite possibilities that the combination of cell/gene therapy seem to offer to the therapeutic arsenal available to humankind.

We must recognise that although enormous progress has been made in our understanding of the biology of stem cells over the last decade, we are still far from completely controlling these systems. For instance, “new players” from the human genome have appeared that were previously unknown; we refer to the small-size and noncoding RNA (known as microRNA) that seem to play an important role in the control of the transcription/translation.<sup>36</sup> Their involvement in human cancer is unquestionable, but their potential role in the biology of ASC is practically unknown. Hence we should be humble and move forward at a rate allowed by the results available from basic research, focusing on taking full advantage of the realistic possibilities at each stage.

Finally, we should note that not everything is rosy in the new universe of stem cells but also that studying them has revealed new concepts that some years ago were pure speculation. It has been shown that some cell populations, originating from the bone marrow but are able to cross into the blood stream in certain circumstances, have an important role in the development of arteriosclerotic lesions, as well as the development of tumour vasculature in various models. The molecular mechanisms that activate this pathological mobilisation of stem cells are not understood in detail. Nevertheless, there are already attempts to exploit this physiological reality to generate new therapeutic options; in particular, the idea of using angioblastic precursors, isolated in peripheral blood or bone marrow, as “cellular carriers” to guide local expression of biomolecules of therapeutic interest in certain areas of the body (secondary tumours, premalignant or arteriosclerotic lesions). These strategies of combined cell-gene therapy have begun to bear fruit at the basic level.



**Figure 4.** Possible relation of ASC with tumorigenic processes. An ASC could suffer a genetic lesion that transforms it into a cancer stem cell (CSC), able to self-renew and generate a tumor containing different cell types. A conventional therapy (1) would destroy most tumor cells, but allowing the survival of a few CSC, which would give rise in time to the formation of another tumor. On the contrary, an hypothetical therapy targeted to the CSC (2) would destroy these cells and the capacity of formation of new tumors. It has not yet fully demonstrated that CSC originate from ASC; they could also derive from other cellular stages partially differentiated (e.g. progenitors) that could de-differentiate or be reprogramed.

Furthermore, an old theory is becoming a clinical reality. Solid tumours are very heterogenic clusters of cells both genetically and functionally. It has been shown in various human solid tumour models that, within the tumours, there is a small population of cells responsible for guaranteeing the properties of the tumour and its aggressiveness (their transplantation in animal models is capable of re-establishing the tumour). This has re-opened discussion of the concept of “cancer stem cells” (CSC), initially proposed from the haematology and oncology fields, and implications of this for the effectiveness of therapies.<sup>37</sup> The truth is that cancer cells and certain populations of stem cells have very similar characteristics and could be related (Fig. 4). In the future, ASC will surely present us with new surprises and, in all likelihood, a new vision of many human diseases.

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## CHAPTER 9

# BONE MARROW TRANSPLANTATION EXTENDS ITS SCOPE

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**Abstract:** The term hematopoietic stem cell transplantation (HSCT) has completely replaced the most widespread bone marrow transplantation (BMT). This semantic change is based on the fact that not only hematopoietic stem cells with capacity for regenerating haematopoiesis and the immune system of the recipient are located in the BM. It was later observed that is possible to mobilise these cells into the peripheral blood, with the aid of certain cytokines, and then collect them through the process of aphaeresis. Moreover, hematopoietic stem cells from umbilical cord blood have been used successfully, and their use in on the increase. The main objectives of HSCT are, first, to substitute a defective haematopoietic system for a healthy one and, secondly, to allow the use of chemo and/or radiotherapy treatment at what would otherwise be supralethal doses, re-establishing haematopoiesis through the administration of haematopoietic progenitor cells. The complications of HSCT tend to be the result of the various factors including toxicity, release of certain cytokine, immunological processes associated with allo-HSCT (especially GVHD) and the effect of immunosuppressive drugs, as we discussed below.

## INTRODUCTION

The first biopsy of bone marrow (BM) described in the literature was carried out by Mosler in 1876, and later, from 1929 onwards, Arinkin advocated bone marrow aspiration as a safe, easy and fruitful approach to the study of haematological diseases. The existence of different cell lines within the BM that were the progenitor of cells

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circulating in peripheral blood (PB) was established once and for all using supravital and Romanowsky staining.

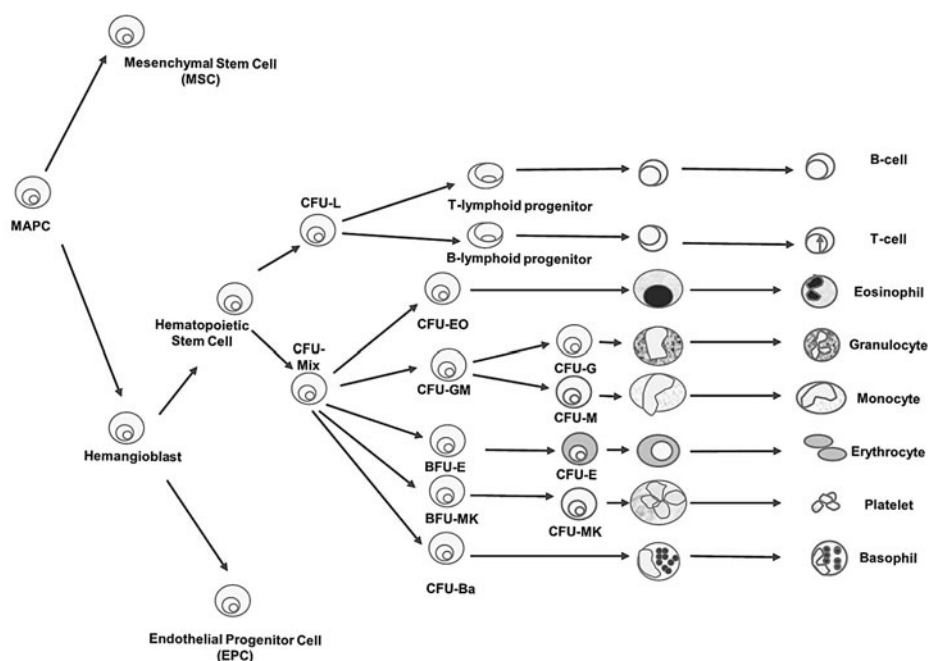
From the nineteen sixties, and thanks to experiments carried out by Till and McCulloch, the existence of one haematopoietic stem cell was recognised.<sup>1</sup> In the nineteen seventies bone marrow stromal stem cells were identified;<sup>2</sup> these came to be called mesenchymal stem cells (MSC) and their current importance will be later discussed in this chapter. In recent years, other types of cells of great interest have also been defined in the BM. These include Multipotent Adult Progenitor Cells (MAPC), initially described by the research group of Dr Verfaillie,<sup>3</sup> which are capable of differentiation towards the cell lineages of all embryonic layers and are considered in other sections of this book, and endothelial progenitor cells, which we will refer to briefly later.

## THE BONE MARROW AS A RESOURCE OF STEM CELLS

### Haematopoietic Stem Cells (HSCs)

Haematopoietic stem cells (HSCs) are the most well studied adult stem cells, and represent approximately 0.05 to 0.5% of the cellularity of the normal BM. As mentioned earlier, it was the experiments initiated by Till and McCulloch<sup>1</sup> in the nineteen sixties that demonstrated the existence of this type of cell, capable of reconstituting the haematopoiesis in lethally irradiated subjects, noting their clonogenic capacity. Given that HSCs are not morphologically identifiable, from the nineteen seventies onwards, various techniques were developed to evaluate their presence functionally, including long-term cultures, delta tests and clonogenic assays. The latter demonstrate the existence of the most committed progenitors. Each progenitor was named in relation to their clonogenic capacity. Hence, the progenitors of the granulomonocytic cells determined by *in vitro* cultures were named CFU-GM standing for Granulocyte/Monocyte Colony-Forming Units, while megakaryocyte precursors became known as CFU-MK, and so on. A diagram of the hierarchical organisation of haematopoiesis is presented in Figure 1.

In parallel with the *in vitro* culture techniques, immunofluorescence and flow cytometry techniques were developed, which have become the usual tool for rapid characterisation of HSCs. While there is no a totally specific marker of HSCs, the antigen CD34, a membrane glycoprotein that is also present in endothelial cells, is the most widely used. Although there are also CD34 negative haematopoietic stem cells in the BM, it is generally assumed that the majority of the progenitors capable of regenerating long-term haematopoiesis are CD34<sup>+</sup>. However, the CD34<sup>+</sup> population present in the BM is a heterogeneous group of cells, including cells that already have their commitment of differentiation towards specific types of haematopoietic cells determined. Our group, using various combinations of monoclonal antibodies, has described the presence of certain CD34<sup>+</sup> subgroups of cells present in the BM committed to lymphoid, erythroid and myeloid cell lines among others, as well as a further subgroup of CD34<sup>+</sup> cells the commitment of which has not been yet determined.<sup>4</sup> As will mentioned later, quantification of CD34<sup>+</sup> cells is of clinical importance, and it is commonly used when collecting haematopoietic progenitors from the BM, mobilised PB and umbilical cord blood (UCB) to be used for HSCT.



**Figure 1.** Hierarchical organization of the bone marrow cell compartments. MAPC: Multipotent Adult Progenitor Cells; CFU: Colony Forming Unit; BFU: Burst Forming Unit.

### Mesenchymal Stem Cells (MSCs)

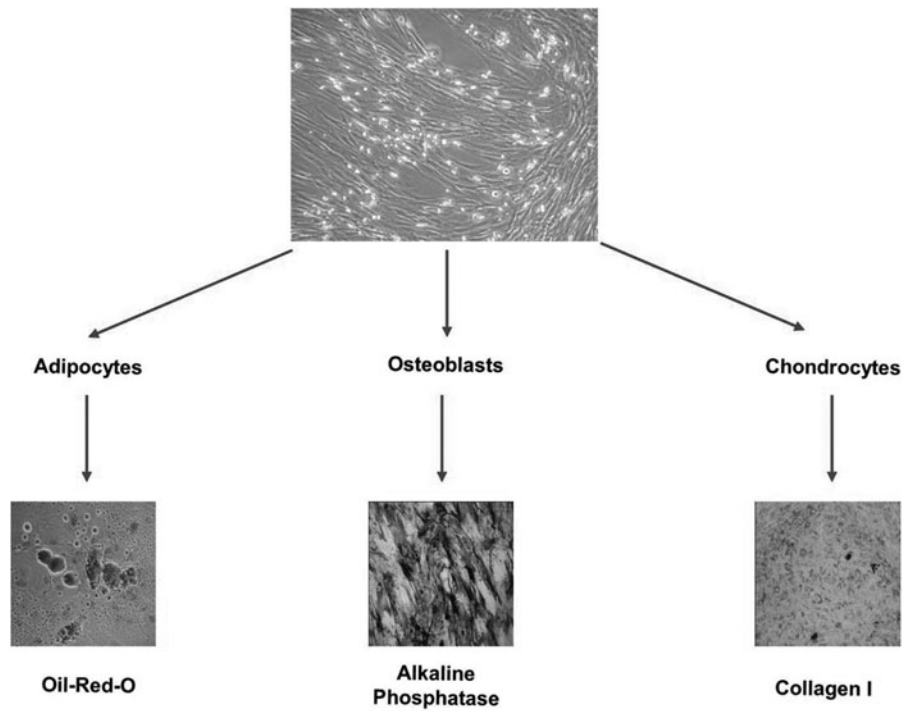
Mesenchymal stem cells are progenitor cells from the bone marrow stroma. The role of this stroma is key for the survival, proliferation and differentiation of haematopoietic cells. It is generally accepted that MSCs were first described by Friedenstein<sup>2</sup> in the nineteen-seventies as fibroblastic progenitor cells with clonogenic capacity (CFU-F), adherence to plastic and capacity for generating osteocytes and adipocytes. Although they have been referred to by different names since their discovery, the consensus now seems to be to use the acronym MSC (from Mesenchymal Stem Cells, or more appropriately, according to the opinion of a panel of experts of the International Society for Cellular Therapy [ISCT],<sup>5</sup> Mesenchymal Stromal Cells). MSCs represent a small percentage of cells in the BM (approximately one in every  $10^4$  or  $10^5$  mononucleated cell), and until now it has not been possible to identify them prospectively, although there are several markers, such as CD271, that can be used to enrich this population. MSCs have a key characteristic in vitro: Adherence to plastic. In culture, MSCs have a fibroblastic appearance exemplified by their fusiform morphology. Since there is no specific immunophenotypic marker for this type of cell, an ISCT working group published a consensus document<sup>6</sup> indicating the minimum characteristics required for an MSC to be considered as such, and these are listed in Table 1. Basically an MSC must: Demonstrate its ability to adhere to plastic; express markers such as CD105, CD90 and CD73; and be negative to other markers, such as CD34 (expressed by haematopoietic stem and endothelial cells), CD45



**Table 1.** Minimum criteria that define mesenchymal stem cells, according to the International Society for Stem Cell Research<sup>6</sup>

1. Adherence to plastic in standard culture conditions		
2. Immunophenotype	Positive (>95% +)	Negative (<2% +)
	CD105	CD45
	CD73	CD34
	CD90	CD14 or CD11b
		CD79α or CD19
		HLA-DR
3. In vitro differentiation: Osteoblasts, adipocytes, chondroblasts (using in vitro staining)		

(pan-leukocyte marker), CD14 and CD11b (monocyte markers), CD19 and CD79α (typical of B lymphocytes) and HLA class 2 antigens (HLA-DR). In addition, MSCs must demonstrate multilinear differentiation, at least towards osteoblasts, adipocytes and chondrocytes, by specific staining of expanded and differentiated cells in vitro in specific differentiation media for each of the three cell types (Fig. 2). Apart from having been isolated from the BM, they have also been obtained from other tissues, including adipose tissue and umbilical cord. The growing fascination with MSCs is due to various properties that make them interesting as potential therapeutic tools. The first of these



**Figure 2.** In vitro multilineage differentiation of mesenchymal stem cells.

characteristics is their capacity for multilinear differentiation. On the basis of this, they have been used in several clinical trials in the areas of traumatology and orthopaedics.<sup>7</sup> Further, these cells have an intrinsic immunotolerance when they are transplanted into HLA-incompatible subjects, even in some models of xenotransplantation. This is an attractive quality; indeed, it would support large-scale in vitro production of these cells for allogeneic use to avoid the need for additional immunosuppressive treatment. Finally, MSCs have great immunomodulatory capacity, with a direct inhibitory effect on T cells in mixed cultures. This enables the scope of their therapeutic potential to extend to diseases with autoimmune basis or to the treatment of graft-versus-host disease in the context of allo-HSCT.<sup>7</sup> We will discuss outcomes achieved in relation to this in a later section of this chapter.

### Endothelial Progenitor Cells (EPCs)

The mesodermal cells that emigrate to the yolk sac early during embryo development are known as hemangioblasts, being common progenitors to both haematopoietic and vascular cells. There are a great number of studies that demonstrate the existence of embryonic hemangioblasts, both in in vitro experiments with embryonic stem cells and in several animal models, especially in *Drosophila*, zebrafish and mice.<sup>8</sup> Conceptually it became accepted that hemangioblasts were a transient cell population, limited exclusively to the embryonic period, which were responsible for vasculogenesis, where by vasculogenesis we refer to the process of formation of blood vessels from primitive endothelial progenitor cells (angioblasts). By contrast, angiogenesis is the formation of blood capillaries from mature endothelial cells of pre-existing blood vessels; it is a response for tissue ischemia and is the main mechanism of neovascularisation in adulthood. However, recent studies have found that there is evidence of vasculogenesis in adults, and therefore that there are angioblasts (vascular progenitors) and even hemangioblasts (common progenitors of haematopoietic and endothelial cells) in postnatal life.<sup>9</sup> In a pioneering study, Asahara et al demonstrated for the first time the existence of circulating endothelial progenitor cells in peripheral blood in humans, based on the expression of CD34 and Flk-1 antigens.<sup>10</sup> Later, the group of Shahin Rafii showed that these endothelial progenitor cells (EPC) expressed the antigen AC133<sup>+</sup> (also known as CD133 or prominin).<sup>11</sup> The antigen CD133, whose biological function is not still well understood, is co-expressed by endothelial progenitor cells and also by hematopoietic stem cells, but not by mature endothelial cells or by myeloid cells, in contrast to the antigen CD34.

Since the initial findings of Asahara et al several research groups have tried to refine the definition of EPCs. However the main problem has been that this cell population does not seem to have a uniform and specific phenotype. Indeed, the majority of the markers that have been used to define EPC, for example, the expression of CD34, CD133, c-kit, VE-cadherin, VEGFR2 and CD31, as well as uptake of Dil-Ac-LDL and *Ulex europaeus* lectin staining, are also co-expressed by other types of cells in the bone marrow and by some cells in the haematopoietic system circulating in peripheral blood, including cells of the myeloid series and monocytes.<sup>12</sup> Nor are there in vitro functional studies which are truly specific for EPCs, for example, as shown recently, the formation of tubular networks when cultured on Matrigel is a property they share with myelomonocytes. The functional test most commonly used in epidemiological studies, namely the formation of colonies in vitro from peripheral blood mononucleated cells in a proangiogenic medium, has been used to associate EPC levels with various prognostic factors of cardiovascular diseases.

However, to add to the confusion, it has recently been found that this test does not effectively assess endothelial cells, but rather that the colonies formed and quantified in this assay are composed of circulating myelomonocytic cells.<sup>12</sup> In this context, we should be really be talking about circulating angiogenic cells, since myelomonocytic cells would exert their action in a paracrine fashion, since it is known that they can synthesise proangiogenic growth factors, such as VEGF (vascular endothelial growth factor), HGF (hepatocyte growth factor) and G-CSF (granulocyte-colony stimulating factor).

However, we should conclude this section by noting that, whether these are really endothelial progenitor cells or proangiogenic haematopoietic cells, what is known today is that the level of the so-called circulating EPC (evaluated either through peripheral blood phenotyping studies [for example CD34<sup>+</sup>/VEGFR2<sup>+</sup>, CD133<sup>+</sup>, etc.] or the number of colonies formed in vitro in a proangiogenic medium) is inversely correlated with risk of cardiovascular events: The lower the level of EPCs, the higher the cardiovascular risk.<sup>13</sup> Also, and despite conceptual discrepancies, preclinical studies in animal models and Phase I-II clinical trials in patients with cardiovascular diseases have shown that the administration of both bone marrow mononucleated cells and selected progenitors, from the bone marrow or mobilised into the peripheral blood (CD34<sup>+</sup>/VEGFR2<sup>+</sup>, CD133<sup>+</sup> and CD133<sup>+</sup>/VEGFR2<sup>+</sup>, among others), improve revascularisation.<sup>14</sup>

## HEMATOPOIETIC STEM CELL TRANSPLANTATION (HSCT)

### Terminology

The term hematopoietic stem cell transplantation (HSCT) has completely replaced the most widespread bone marrow transplantation (BMT). This semantic change is based on the fact that initially it was thought that hematopoietic stem cells with capacity for regenerating haematopoiesis and the immune system of the recipient were only located in the bone marrow (BM). However, it was later confirmed that it was possible to mobilise these cells into the peripheral blood, with the aid of certain cytokines, and then collect them through the process of aphaeresis. Indeed, this has become the most commonly used procedure today. Finally, hematopoietic stem cells from umbilical cord blood have been used successfully, and their use is on the increase. For these reasons, instead of continuing to refer to the source of cells used at the start, namely the bone marrow, it seems more appropriate to now call the procedure HSCT.

### Historical Outline

The basis of the initial development of HSCT arose following the Second World War in the context of study and prevention of the effects of radiation on the body. It was demonstrated then that the BM was the system component which is most sensitive to these effects, and that the result was bone marrow insufficiency that in many cases is fatal. Jacobson et al confirmed that by protecting the spleen (one of the primitive haematopoietic organs), mice that underwent total body irradiation (TBI) could survive the procedure. Later, Lorenz et al observed an identical protective effect with bone marrow infusion. From 1955, the Seattle group, led by professor E. Donnall Thomas (later awarded with the 1990 Nobel Prize), started to lay the foundations for HSCT using their canine model, highlighting for the first time the importance of the Major Histocompatibility Complex

and describing Graft-versus-host disease (GVHD). Thomas et al carried out the first transplant with TBI in twins suffering from acute leukaemia in 1959. In Europe, the group of Mathé in France reported the first case of acute leukaemia to survive bone marrow transplantation in 1963. In Spain, the first allogeneic transplants were carried out 1975 in the Clinic Hospital and Sant Pau Hospital, both in Barcelona. Since then, transplant activity has been progressively increasing, more than 25,000 transplants being carried out every year in Europe (10,000 allogeneic and 15,000 autologous), according to the European transplant registry (European Group for Blood and Marrow Transplantation, EBMT).<sup>15</sup>

### **Physiological Bases of HSCT and Types of Transplant (Autologous and Allogeneic)**

There are two main objectives of HSCT: To substitute a defective haematopoietic system for a healthy one and, secondly, to allow the use of chemo and/or radiotherapy treatment at what would otherwise be supralethal doses, re-establishing haematopoiesis through the administration of haematopoietic progenitor cells.

This type of chemotherapy and radiotherapy regimen is termed conditioning, and eliminates all the haematopoietic elements (normal and abnormal, if present) from the bone marrow. As already mentioned, the re-establishment of the haematopoietic activity is achieved through the administration of haematopoietic progenitors from a healthy donor or from multiple units of umbilical cord blood (in the case of allogeneic transplantation, allo-HSCT), or coming from the same patient (known as autologous transplantation, auto-HSCT).

The basis of the autologous transplant is to make it possible to administrate high doses of chemotherapy and/or radiotherapy to a patient to try to control cancer. In this sense, it would be possible to consider self-HSCT as a “cycle” rather than chemotherapy. Nonetheless, the doses administered in this case are myeloablative, they mean that the recovery of the haematopoietic function is not possible unless the progenitors, previously extracted from the patient and cryopreserved, are re-administered to the patient after the conditioning treatment.

In the case of allo-HSCT, progenitors cells can come from the BM or from the peripheral blood (PB) mobilised using haematopoietic growth factors, principally G-CSF (Granulocyte-Colony Stimulating Factor), of HLA-compatible individuals, related or not. Another possibility is to obtain the progenitors from the umbilical cord blood, again with the necessary degree of HLA-compatibility. In all cases, in allo-HSCT, the progenitors do not come from the transplanted individual, and therefore there immunological interactions between the cells from the two sources. These interactions are of a different kind to those that occur in solid organ transplants and are a key differential characteristic of allo-HSCT. In solid organ transplantation, the transplanted organ contains a small number of cells with immunological functions, such that the immune system after the transplant continues to depend on the BM of the transplanted patient (the host). For this reason, life-long administration of immunosuppressive drugs is necessary to avoid the rejection of the transplanted organ, and indeed this is the main complication in this type of transplants. However, in allo-HSCT the conditioning treatment prior the transplant with myeloablative chemo- and radiotherapy destroys most of the cells of the immune system of the patient, and it is precisely the transplanted organ (in this case the bone marrow), which is responsible producing not only the haematopoietic cells but also all the cells make up the immune system of the receptor. For these reasons,

in allo-HSCT, the rejection of the graft, although possible is less common problem than in solid organ transplantation, the main complication being Graft-versus-host disease (GVHD), which occurs when the immunocompetent cells coming from the BM of the donor recognise as foreign various organs or tissues of the recipient. The aim of immunosuppressive treatment administered to patients who undergo allo-HSCT is to prevent the development of GVHD and, unlike in solid organ transplantation, in most cases it can be discontinued in the long-term, immune tolerance from the graft to the host having been achieved. An interesting point to note is that GVHD is not only associated with adverse effects. Several years ago the first retrospective studies of the various international registers of patients who underwent allo-HSCT observed an association between the development of GVHD and a smaller risk of recurrence of the post-transplant haematological disease.<sup>16</sup> In this sense, the curative effect of allo-HSCT is not only due to chemo and/or radiotherapy employed in the pretransplant conditioning, but also to this “Graft-versus-leukemia” (GVL) effect contributing to further treat the disease through the immunological recognition and the destruction of residual tumour cells that may persist in the patient. Other clinical reports have fully confirmed these findings and, likewise, the depletion of T cells both *in vitro* and *in vivo*, a technique which may be used in the post-transplant period used to prevent the development of GVHD, is associated with a higher risk of post-transplant recurrence.<sup>16</sup> Moreover, patients who after allo-HSCT suffer a relapse of certain diseases, the clearest example being chronic myeloid leukaemia (as we will see later), can be successfully treated by infusion of T cells from the same donor.

### Source of Cells

As specified earlier, the first HSCTs were carried out from progenitors obtained directly from the BM. Carried out in the operating theatre under general anaesthesia and aseptic conditions, the technique consists of BM aspiration by multiple punctures (between 100 and 200 of between 3 and 5 ml each) in the anterior superior iliac crest areas, and if necessary from the posterior. With this method between 800 and 1200 ml of BM blood (a maximum of 15 ml/kg of weight) is obtained from an adult. The final product tends to have between 10 and  $20 \times 10^9$  total nucleated cells (approximately  $1.5\text{--}3.5 \times 10^8$  nucleated cells/kg and  $2\text{--}3 \times 10^6$  CD34<sup>+</sup> cells/kg). As the bone marrow is extracted, it is collected in a transfer bag containing an anticoagulant solution (generally ACD or heparin). Once the extraction is finished, the product is sieved (with 500 and 200  $\mu\text{m}$  pore filters) to remove lumps of marrow and chips of bone. In general, given the volume extracted, the donor is given an autotransfusion of red blood cells, from blood taken at least three weeks before the procedure. The most severe complications resulting from the extraction of BM (e.g., pulmonary embolism and side effects of anaesthesia) occur in less than 0.3% of cases. However, there are mild complications (mainly pain) in at least 10% of cases.<sup>17</sup>

After the first BM transplants, it was observed that under certain clinical conditions (such as in the recovery from post-chemotherapy aplasia and the administration of haematopoietic growth factors) and only during a certain time window, a considerable number of haematopoietic progenitors were mobilised from the BM to PB. For the donation of PB progenitors, donors (or patients, in the case of self-HSCT) receive in general G-CSF at doses of 5–10  $\mu\text{g/kg}$  every 12 hours for 4 days, after which they undergo leukapheresis. During this procedure, which takes 2–3 hours, the donor is

connected to a cell separation device with blood being extracted from one vein and returned to another. During the procedure, around 10–20 litres circulate through the system and the mononucleated fraction (which contains CD34<sup>+</sup>) is collected in a bag, while the neutrophils, red blood cells, platelets and plasma are returned to the donor. The number of CD34<sup>+</sup> cells obtained through leukapheresis after mobilisation is higher than that obtained from BM extraction, and the cell content is also different, since it contains approximately 10 times more T cells. Both of these aspects are important: The first means that the haematopoietic graft must be carried out more rapidly than a BM transplantation; and the second means that it also entails higher risk of suffering from GVHD, since T cells from the donor are the main players in this complication.

Despite there currently being more than 11 million haematopoietic donors (BM and PB) on registers worldwide,<sup>18</sup> it is sometimes difficult for certain patients to find compatible HLA donors, due to the great polymorphism of the HLA system. For this reason, there has been a search for alternative sources of haematopoietic progenitors, such as umbilical cord blood (UCB). Currently UCB is the third source used in practice (the first HSCT of UCB was carried out in 1988) but it is of growing importance.

UCB is very rich in haematopoietic progenitors, and the lower immunological reactivity of the lymphocytes allows there to be some degree of HLA incompatibility with the recipient, correspondingly, the incidence and intensity of GVHD is lower in patients who have received a UCB transplant. The collection of UCB can be undertaken before delivery of the placenta (in utero collection) or afterwards (ex utero), the second being the most common technique. Following the birth, the umbilical cord is clamped as close as possible to the baby. After cord ligation, the umbilical vein is punctured with a needle attached to collection bag in which the blood of the cord is deposited by gravity (in general between 40 and 150 ml are obtained). After analysis of the microbiology, serum and HLA system, the CD34<sup>+</sup> cells are quantified, and then the unit of UCB is cryopreserved until used. At present there are more than 300,000 units of UCB in international transplant registries.<sup>18</sup> Table 2 shows the advantages and disadvantages of the use of mobilised PB and UCB with respect to BM as sources of haematopoietic progenitor cells for transplants. In practice, the data from the Spanish National Transplant Organization (ONT) show that of the 314 allo-HSCT from nonrelated donors (excluding therefore self-HSCT and transplants between HLA-identical siblings) carried out in 2008 in Spain, BM was used as a source of cells in only 59 cases, mobilised PB was used in 120 cases, while UCB was used 135 cases (most of these in paediatric patients).<sup>19</sup>

**Table 2.** Main advantages and disadvantages of the use of PB and UCB compared to BM as a source of haematopoietic progenitors for transplantation

Source	Advantages	Disadvantages
PB	<ul style="list-style-type: none"> <li>• Avoids general anaesthesia</li> <li>• Faster recovery of haematopoiesis</li> </ul>	<ul style="list-style-type: none"> <li>• Higher occurrence of GVHD</li> </ul>
UCB	<ul style="list-style-type: none"> <li>• Rapid availability</li> <li>• Less stringent requirements with respect to HLA compatibility</li> <li>• Lower risk of GVHD</li> </ul>	<ul style="list-style-type: none"> <li>• Limited cell doses</li> <li>• Neither a second donation nor the administration of donor lymphocytes are possible</li> <li>• Slow engraftment</li> </ul>



## Main Complications

The complications of HSCT tend to be the result of the various factors including toxicity associated with the conditioning treatment, the release of certain cytokines that cause cell damage, immunological processes associated with allo-HSCT (especially GVHD) and the effect of immunosuppressive drugs.

The signs and symptoms of early toxicity resulting from the conditioning treatment include nausea, sickness, diarrhoea, mucositis, haemorrhagic cystitis (generally associated with the administration of cyclophosphamide or due to certain viral infections) and alopecia.

There are also multifactorial complications, such as infections (which are common and can be severe), hepatic veno-occlusive disease, thrombotic microangiopathy and idiopathic interstitial lung disease.

Immunological complications include graft rejection (not common), and GVHD, which has been already discussed earlier when describing the pathophysiological bases of the transplantation. These complications are exclusive to allo-HSCT. Autoimmune disorders can also develop, in particular cytopenia.

Finally, there are late complications among which the following should be highlighted: Endocrine disorders (thyroid, growth disorders, osteoporosis, etc.), sterility, neurological disorders (leukoencephalopathy), cataracts, or the development of secondary tumours.

## Current Indications for HSCT

HSCT has been considered as a possible therapeutic approach for numerous different diseases. Recently, the EBMT has analysed the activity of autologous and allogeneic HSCT for the treatment of haematological diseases, solid tumours and immune disorders in Europe.<sup>20</sup> The indications are classified under four categories: S: Standard of care (standard treatment), generally indicated in suitable patients; OC: Clinical option, can be carried out after an adequate assessment of risks and benefits; D: Developmental, further clinical trials are needed; and GNR: Generally not recommended. Table 3 provides a list of the diseases for which HSCT can be used. For more detailed information, consult the paper published by the EBMT working group.<sup>20</sup>

## POST-TRANSPLANT IMMUNOTHERAPY

Two aspects should be addressed before considering post-transplant immunotherapy. First, immunotherapy for the prevention or treatment of infectious processes after transplantation, mainly against Cytomegalovirus (CMV), the infection of which remains one of the main causes of morbidity and mortality following HSCT. It has been demonstrated that the re-establishment of the T-cell response against post-transplant CMV prevents the development of the disease caused by this agent. Second, we will mention the role of immunotherapy in the treatment of the underlying disease, the best example of this being donor lymphocyte infusion (DLI).

It has been known for some years, that a substantial number of T cells, both CD4<sup>+</sup> and CD8<sup>+</sup> specific to CMV can be detected in healthy CMV-seropositive individuals, and that these cells mediate the control of viral reactivity.

Several studies have demonstrated the feasibility of obtaining and administering CMV-specific T cells from the donor, both fresh preparations and after *in vitro* expansion.



**Table 3.** Diseases for which therapy with some type of HSCT treatment could be considered under certain clinical circumstances

Haematological diseases	Acute myeloid leukaemia
	Acute lymphoblastic leukaemia
	Chronic myeloid leukaemia
	Myeloproliferative syndromes
	Myelodysplastic syndromes
	Chronic lymphoid leukaemia
	Non-Hodgkin lymphoma
	Hodgkin's disease
	Multiple myeloma
	Bone marrow aplasia
	Primary amyloidosis
	Paroxysmal nocturnal haemoglobinuria
	Thalassaemia
	Sickle cell anaemia
	Fanconi anaemia
	Diamond-Blackfan anaemia
	Primary immunodeficiencies
Solid tumours	Breast cancer
	Germ cell tumours
	Ovarian cancer
	Glioma
	Small cell lung cancer
	Renal cell carcinoma
	Ewing's sarcoma
	Soft tissue sarcoma
	Neuroblastoma
	Wilms' Tumor
Systemic diseases	Osteogenic sarcoma
	Brain tumours
	Systemic sclerosis
	Rheumatoid arthritis
	Lupus erythematosus
Diseases caused by deposits	Crohn's disease
	Multiple sclerosis
	Hurler's syndrome (Mucopolysaccharidosis 1-H)
	MPS 1-H Hurler-Scheie
	MPS-VI Maroteaux-Lamy
	Osteopetrosis
	Other diseases caused by deposits

Another approach has been the activation and in vivo expansion through stimulation with antigen-presenting cells, in particular dendritic cells, loaded with specific proteins and CMV peptides. However, this last procedure is time-consuming and expensive. According to the clinical experience accumulated over recent years, there are several potential

strategies for immunotherapy against CMV. First the Seattle group<sup>21</sup> demonstrated that the administration of CMV-specific CD8<sup>+</sup> T-cell clones did not cause side effects and contributed to the control of infection by CMV. However, the process of expansion of the clones required up to eight weeks of culture. The German group lead by Professor Einsele has actively worked in the development of several types of immunotherapy against CMV.<sup>22</sup> One of these involves the administration of CMV-specific polyclonal T cells, which also require in vitro expansion for several weeks. More recently, this group has published its results regarding infusion of antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes enriched through a process carried out under GMP conditions in just 10 days and from a sample of 500 ml of PB from the donor. Finally, the progress with regards to the development of peptide-HLA multimers has allowed antigen-specific T cells to be isolated, using immunomagnetic separation and flow cytometry. A new type of HLA-multimeric complex that binds reversibly to the T-cell receptors, make it possible to obtain CMV-specific T cells rapidly (an almost “fresh isolate”, with no need for a lengthy culture process). In this way, the risks are minimised and the costs of in vitro expansion avoided.

With respect to DLI, this is the best example of the therapeutic effect of alloreactive donor T cells. The paradigm is chronic myeloid leukaemia, in which DLI enables approximately 60-70% of patients who suffer a relapse after allo-HSCT to make a complete recovery, even at the molecular level (negative BCR-ABL using RT-PCR). Other blood diseases, such as lymphoproliferative syndromes and multiple myeloma, are sensitive to DLI, although to a lesser extent. Further, despite the beneficial effect of DLI, this procedure is not free of risks. The most common problem is GVHD, and this occurs in almost half of patients. The incidence of GVHD after DLI can be decreased by carrying out infusions with increasing doses of lymphocytes or by reducing the number of CD8<sup>+</sup> lymphocytes present in the sample, although this may, evidently, decrease the efficacy of the procedure.<sup>15</sup> There have been several attempts to administer donor T cells transduced with a suicide gene, in order to be able to eliminate them if the patient develops GVHD. The most notable example of this is the thymidine kinase gene of the herpes simplex virus (HSV-TK) that makes the transfected cells prone to the action of ganciclovir. However, despite the promising results obtained, this therapeutic strategy is still far from clinical application.<sup>15</sup>

## POTENTIAL OF MESENCHYMAL STEM CELLS FOR HSCT

As indicated previously, MSCs have various properties that make them very attractive to for clinical use. One of the first fields in which they have been applied in the context of clinical trials has been, as could be expected, HSCT. The role of MSCs in HSCT can be viewed in two ways: On the one hand, as a promoter of the haematopoietic graft, given their function as bone marrow stromal progenitors, while, on the other hand, as treatment a of HSCH, given their immunomodulatory properties.

Research on bone marrow xenotransplantation has demonstrated that the cotransplant of MSCs favours the grafting of haematopoietic progenitors in immunosuppressed mice. Further, in a pilot study with 28 breast cancer patients, who underwent self-HSCT, the administration of MSCs favoured the autologous haematopoietic graft with no adverse effects.<sup>23</sup> More recently, the group at the Karolinska Institute has reported an experiment in a small series of patients with graft failure after HSCT who received MSCs and subsequently achieved remission of their cytopenia.

With respect to the use of MSCs as a treatment of GVHD, the Swedish group was the first to publish the results of the first patients treated, and recently this group has published the first results of a Phase II trial using MSCs in 55 patients with steroid-resistant acute GVHD with good outcomes,<sup>25</sup> and with no notable adverse effects during the administration of cells. Randomized Phase III clinical trials are being carried out in order to confirm the efficacy of MSCs in this context.

## **HSCT AS A BASIS AND MODEL FOR SOMATIC CELL THERAPY IN REGENERATIVE MEDICINE**

Most of the advances achieved in recent years in the field of stem cells and regenerative medicine have been based on haematopoietic progenitor cell transplantation. The first donor cells of phenotype different to the BM and localised in various different organs and tissues were detected in women who underwent allo-HSCT from male donors. Based on the experience of several years with HSCT and a deep understanding of the types of cells within the BM, the first clinical trials carried out in the field of regenerative medicine, mainly in the cardiovascular field, have used cells obtained from the BM in similar conditions to those obtained for HSCT. Even the steps for mobilizing progenitor cells with G-CSF are the same. The techniques for the analysis of progenitor cells and the detection of the graft are also based on previous work carried out in the context of allo-HSCT. Further, HSCT itself has started to benefit from the therapeutic use of other types of cells, such as MSCs. Given all this, there is no doubt of the current importance of HSCT and it is certain that it will continue to be key in the progress in cell therapy and regenerative medicine in coming years.

## **CONCLUSION**

Bone marrow has been the first source of stem cells (hematopoietic stem cells) successfully employed in the clinical setting for almost 50 years. In addition, it contains other stem cell subsets (mesenchymal stem cells and endothelial progenitors) that are potentially of use in regenerative medicine and that are the basis of most clinical trials currently active in this field.

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## CHAPTER 10

# BIOLOGY OF STEM CELLS: The Role of Microenvironments

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**Abstract:** From the discovery of the first line of human embryonic stem cells, thousands of studies have been published concerning adult stem cells and their possible alleged therapeutic potential. However, very little real progress has been made in the application of cell therapy to patients. We can conclude that there remains a great deal for us to learn about the biology of stem cells, and especially, the mechanisms that regulate their differentiation and use under conditions of biosafety. In this chapter, we are going to review some of the mechanisms that seem to control the biology of stem cells, in particular the microenvironments, also called niches, where they house and which exert a strong influence over them. The regulation, survival, proliferation and differentiation of stem cells is ultimately determined by a combination of factors intrinsic to the stem cells themselves and extrinsic signals received from the microenvironment. A better understanding of the cellular components of microenvironments and their cellular and molecular interactions with the other components of the niche, including the stem cells themselves, will be key to make progress in this field.

## INTRODUCTION

Since the first cell lines of human embryonic stem cells were obtained in late 1998, and there was evidence that implied, though did not yet demonstrate, that stem cells existed in adult tissues that were able to produce, directly or indirectly, cells from other lineages, thousands of studies have been published concerning adult stem cells and their possible alleged therapeutic potential. However, very little real progress has been made in the application of cell therapy to patients. The conclusion we can draw, without

ignoring the clear potential of these therapies, is that there remains a great deal for us to learn about the biology of stem cells, and especially, the mechanisms that regulate their differentiation and use under conditions of biosafety.

In the next few pages we are going to review some of the mechanisms that seem to control the biology of stem cells, in particular the microenvironments, also called niches, where they house and which exert a strong influence over them. It is very likely that the regulation, survival, proliferation and differentiation of stem cells is ultimately determined by a combination of factors intrinsic to the stem cells themselves and extrinsic signals received from the microenvironment. In relation to this, asymmetry is a common phenomenon, although not universally linked to the control of the biology of stem cells. By asymmetry we mean the possibility of differential segregation of the cellular components and/or molecules to produce two daughter cells with different characteristics. This is not necessarily an asymmetric division producing two daughter cells of different sizes and located in different locations within the niche, although this is the most common case.

Accordingly, we can speak about mechanisms intrinsic to the stem cells themselves that can organise their organelles and/or molecules, inducing intracellular polarity and thereby great distinct biological potential to two regions within the cell, and about extrinsic mechanisms that affect stem cells with the same potential in different ways.

## **INTRINSIC CONTROL MECHANISMS OF SURVIVAL AND DIFFERENTIATION OF STEM CELLS**

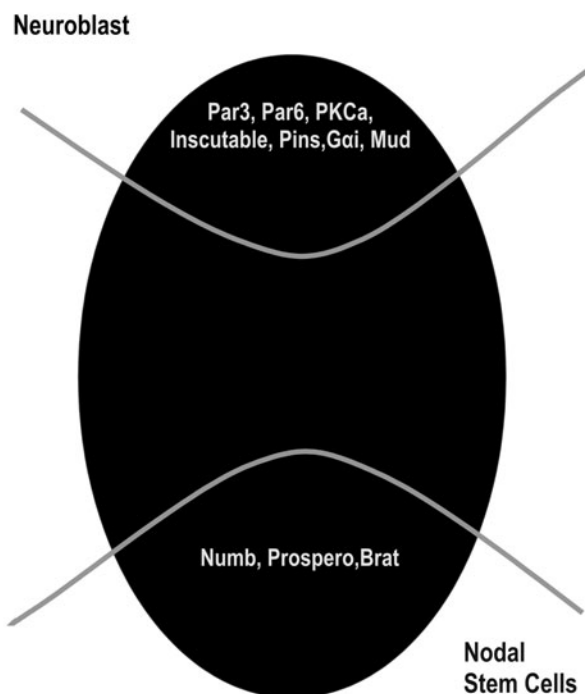
### **Asymmetry and Differential Segregation in Invertebrates**

Asymmetries within stem cells have been found, for example, in *Drosophila* in the progenitors of both central nervous system and sensory organ (peripheral nervous system).<sup>1,2</sup> Peripheral progenitors divide leading to one anterior and one posterior cell, which in turn produce two external and two internal cells of the sensory organ. In all these division cycles, the generation of asymmetry depends on the different levels of Notch in both cells. As far as neuroblasts are concerned, they undergo several cycles of division each of which generating two different-sized cells: The largest one remains a neuroblast while the smallest one becomes a ganglion stem cell that divides only once to give two neurons. On the other hand, after the division, these neuroblasts follow one of two different behaviours according to whether they are embryonic, those that generate the larval nervous system, or larval, those that give rise to the adult nervous system. In the first type, the cells arising from each cell division are progressively smaller, while in the larval type, after each division, the resulting cells grow to reach the original size.

These different behaviours are caused by the differential segregation of the various proteins in one pole or the other of the dividing cell. Par-3, Par-6, aPKC (atypical), Inscuteable (Insc), Pins, G $\alpha$ i and Mud accumulate in the apical pole, while in the other pole, which will turn into a ganglion stem cell, there is an accumulation of Numb, Prospero (Pros) and Brat, which are considered the real determinants of the asymmetry (Fig. 1). Although the underlying mechanism for the asymmetric segregation is still not well understood, we will now consider some of the data available.

The key substrate of aPKC is a cytoskeleton protein called Lg1 (lethal giant larvae), that is necessary in epithelial cells to specify the basolateral domain and to restrict aPKC, Par-3 and Par-6 to the apical domain. On the other hand, in embryonic

## Segregation of determinants in *Drosophila* neuroblasts



**Figure 1.** Segregation of determinants in *Drosophila* neuroblasts.

neuroblasts, while Lgl is not required to localise these proteins on the apical side, it is necessary to recruit the determinants of the cell cortex and arrange them asymmetrically during mitosis. Insc, given its association with Par-3, is found apically and draws to this location other proteins known as Pins, the carboxyl-terminal of which have three GoLoco domains that bind the subunit G $\alpha$ i of the heterotrimeric G proteins. The binding to the first domain results in the recruiting of Pin to the plasma membrane, facilitating their apical concentration. On the other hand, the binding to the second and third domains induces a conformational change in the amino-terminal of the molecule, enabling it to interact with Mud (a homologue of the protein NuMA that binds to microtubules and dynein). This cascade of interactions presumably gives an “anchor point” through Mud for microtubules that will attract a pole of the spindle and will determine its polarization. Some data show that this Insc/Pins/G $\alpha$ i molecular complex acts in different ways in embryonic and larval neuroblasts. In the former, in an Inscutable-dependent process, the mitotic spindle rotates during the metaphase in any of the two directions, suggesting that any of the centrosomes could determine the apical pole of the spindle. On the other hand, in larval neuroblasts, it is always the “oldest” centrosome which is closer to the Insc/Pins/G $\alpha$ i complex, while the new centrosome first migrates randomly within the cell and later fixes the basal pole of the spindle.



As for the determinants that segregate in the ganglion stem cell, Numb is a tissue specific inhibitor of the Notch signalling pathway, which binds to the endocytic protein alpha adaptin, and controls the intracellular “traffic” of the Notch mediators. When Numb mutates in the larval brain, neuroblasts hyperproliferate and form tumours that remain, both phenotypically and potentially, neuroblasts.

Prospero is a transcription factor that, after entering the nucleus, commits the ganglion stem cells to dividing neuroblasts. The absence of Pros in embryonic neuroblasts causes the ganglion stem cells to continue to divide while maintaining markers of neuroblasts. In the larval neuroblasts, the same mutation causes the appearance of stem cell tumours. Pros binds to many of the genes that are involved in self-renewal or control of the cell cycle of the neuroblasts, but may also induce the expression of differentiation genes working as transcriptional activators and inhibitors. The Brat gene, like other genes of the same family (Mei-P26, Dappled), is a tumour suppressor gene that co-operates with Pros in the specification of the phenotype of ganglion stem cells. While the absence of Pros only affects a few ganglion stem cells, Pros/Brat double mutants completely lack these stem cells.

The mechanisms that assure the asymmetric segregation of Pros, Brat and Numb are mediated by two “adapter proteins”, Miranda and PON (Partner of Numb). Miranda is a molecular adapter required to connect Brat and Pros to the machinery for asymmetric distribution of proteins. In Miranda mutants, Pros and Brat segregate equally to both neuroblast daughter cells. The Numb adapter is PON but in this case it is not essential for their asymmetric distribution. That is, in PON mutants, though the asymmetric distribution of Numb is delayed it does eventually occur, however this delay causes alterations in the division of the neuroblasts.

### **Asymmetries and Differential Segregation in Mammals**

The segregation of determinants of mammalian stems cells has been studied much less, mainly due to the fact that they are few in number and their identification in tissues is difficult. Further, they also have long life cycles and/or are even quiescent in physiological conditions.

The orientation of the spindle seems to influence the commitment of the daughter cells during mouse neurogenesis. Such orientation requires, as in *Drosophila*, heteromeric G proteins and their binding proteins Pins and Inscuteable. When those G proteins are inhibited or the Pins (AGS-3) homologues are blocked with small interfering RNA, the number of symmetric divisions of the radial glial cells increases. Further, proteins such as Par-3 (called ASIP in vertebrates), Par-6 and aPKC (that in vertebrates correspond to the isoforms PKC $\zeta$  and PKC $\lambda$ ) are located apically in the neural progenitors, concentrating in the adherens junctions (zonula adherens). When a single isoform of aPKC mutates, the adherens junction disassembles, but the polarity of the neuroepithelium is maintained and the neurogenesis is carried out normally. On the other hand, in the absence of Par-3 or Par-6, the progenitor cells differentiate, while the over-expression of Par-6 increases the level of proliferation and the number of symmetric divisions.

Mouse homologues of the other determinants described in *Drosophila* have been identified, but their role in neurogenesis has not yet been established. Prox-1 (homologue of Pros) and TRIM 2, TRIM 3, TRIM 32 (homologues of Brat) are expressed in the brain, but they do not seem to segregate asymmetrically and their role in neurogenesis is not clear. Numb and Numb-like (homologue of Numb) work redundantly inhibiting the Notch

signalling pathway; Numb-like is distributed across the cytoplasm while Numb is only found apically. When both molecules are deleted in neural progenitors, they decrease in number progressively until neurogenesis stops. However, in Numb-like deficient mice, the proliferation of the progenitors increases, and the absence of Numb does not affect adult neural progenitors. So, the role of these molecules in neurogenesis is far from clear. It is possible that they are not essential proteins (as they are in *Drosophila*) or that their effects are not direct. For example, it has been suggested that the function of Numb may be to maintain the adherent junctions of the radial glia that surround dividing progenitors and regulate the E-cadherin traffic. Its absence would alter the junctions which, in turn, would affect the survival and proliferation of the neural progenitors. We also should not rule out that in mammals there are other molecules involved in asymmetric segregations of the determinants, which might not have been identified in *Drosophila* and other nonmammals.

### EXTRINSIC MECHANISMS OF CONTROL OF THE MAINTENANCE AND DIFFERENTIATION OF STEM CELLS

After considering the role played by the differential segregation of organelles and molecules in the determination of the commitment of stem cells we will review the concept of microenvironment (or niche) and its role in the generation of external signals that cause cells with identical potential to behave differently. In particular, we will examine in depth the nature of these signals and their maintenance during evolution using different, more or less complex models of niches to understand their organisation and consider the numerous unanswered questions concerning their biology.

The niches are physiologically-defined microenvironments with properties to regulate and support stem cells, capable of controlling the balance of quiescence, self-renewal and differentiation, largely through cell-cell interactions. The best characterised niches are those of the sexual organs of *Drosophila* and *Caenorhabditis*. In mammals, those of the hair follicles, the intestinal crypts of Lieberkühn, the neurogenic areas of the central nervous system and, in particular, the haematopoietic bone marrow are relatively well known, although the results to date are much more controversial.<sup>3</sup> In all of the aforementioned examples the niches vary in size and complexity.

The niches of the cysts of *Drosophila* ovarioles house only one somatic stem cell, namely a follicle stem cell, but niches at the tip of the ovariole and the testis are larger and contain more types of cells. The ovarian niche normally contains 2 or 3 germ line stem cells and between 4 and 8 accompanying somatic cells, as well as the 4 to 7 cap cells and those that determine the axis of the ovariole (hub cells), while the testicular niche contains 10 to 15 germinal stem cells and between 20 and 30 cyst progenitors. In the gonads of *Caenorhabditis* there are up to 50 germ line stem cells. All these small niches are typical of invertebrates while in mammals there are many stem cells within relatively small structures, such as the hair root and the subventricular zone of the brain, as well as in much larger regions such as the bone marrow. The exception is the skeletal muscle where satellite cells, a population of cells that includes muscle stem cells, are located individually at random beneath the basement membrane of the muscle fibres. Further, in the majority of cases, both in invertebrates and vertebrates, the niches remain in the same place through the life, but the system is much more dynamic in the bone marrow, with the haematopoietic stem cells constantly changing or occupying various locations within the bone marrow according to their activation state (see later).

### The Niches of the Gonads of *Drosophila* and *Caenorhabditis*

In both sexes, the functioning of the gonadal niche of *Drosophila* is based on the interactions between the germ line stem cells and the niche cells, to which they bind to by adherent junctions, in such a way that the former receive local signals from the latter if they keep together, but not if they separate. In this way, the cells closest to the niche will retain their status as stem cells while those located distally will start to differentiate. The signalling molecules and the underlying mechanisms used are, however, slightly different in the niches of the ovariole and the testis.<sup>4</sup>

In the case of germ line stem cells of the *Drosophila* ovary, the process is dependent on the bone morphogenetic proteins (BMP) signalling. The somatic niche cells (cap cells) express ligands known as decapentaplegic and GBB (glass bottom boat) of the BMP-Type I and II receptors (called Tkv and Sax in *Drosophila*) expressed by the germ line stem cells. The activation of the BMP signalling pathway represses the transcription of Bam (bag of marbles) in these cells, a key regulator for the differentiation that is deactivated in germ line stem cells. When these cells divide, one of the two daughter cells loses the BMP signalling pathway and initiates the transcription of Bam, which works with BGCN (benign gonial cell neoplasm) to drive the daughter cells to leave the niche. The daughter cells then start to differentiate, and this is stabilised by the activation of d-smurf, an E3 ubiquitin ligase that blocks the residual BMP signalling pathway, promoting the degradation of the SMAD, Medea, involved in the mentioned signalling.

In the case of male niches, the situation is slightly different, although the “key players” are similar. The control of the self-renewal of the stem cells is initiated by other ligand, UPD (unpaired) which is expressed by the hub cells and activates the Jak-Stat signalling pathway. However, the BMP signalling pathway is also necessary to maintain the germ line stem cells, via, once again, the repression of Bam. Here its ligand, GBB is not only expressed in the hub cells but also in the cyst cells, and repression of Bam occurs in the gonioblasts as well as in the germ line stem cells. Bam and BGCN work in this case, stopping, rather than initiating, the mitotic cell divisions of the cyst progenitors undergoing differentiation. Accordingly, the Jak/Stat is the main signalling pathway in the tip of the testicular niche, later giving way to that mediated by BMP.

In addition, in both sexes, germ line stem cells bind to the niche cells by adherent junctions rich in E-cadherin; indeed, its disappearance results in loss of the germ line cells. However, the mechanisms for maintaining such cells within the niche are different: In males, APC2 (anaphase-promoting complex 2), involved in the orientation of the mitotic spindle, is located along with E-cadherin in the cortical area where the stem cells bind to the hub cells and orient the spindle perpendicular to the niche axis. The “oldest” centrosome is also located in this region; the newest one migrating to the opposite site of the germinal stem cell. There is no evidence of this occurring in the female, where it may simply be the lack of space within the niche that makes the daughter cells to leave it and start to differentiate.

The gap junctions seem to play also a role in the control of germ line stem cells in *Drosophila*.<sup>5</sup> In particular, mutant cells in the Zpg (zero population growth) gene that encodes for innexin 4, specific of the gap junctions of the germ line stem cells, are viable but sterile. The gonads only contain a few primitive germ line stem cells which are not able to progress to more advanced stages. Zpg is located on the surface of the spermatogonia and presumably mediates their interaction with the somatic cyst cells. In females, Zpg

is located at the interface between the niche cells and germ line stem cells as well as on the surface of the most mature germinal cells.

The *Drosophila* niches are even more complex and, therefore, need additional signals of which only a few have so far been identified. For example, the accompanying cells which are located in the sheath surrounding the germarium make contact with the cap cells and germ line stem cells, and also divide asymmetrically participating in cyst production so that cystoblasts and individual cysts are located between the cell processes of their progeny. The biology of the shield cells seems to depend on the Jak-Stat pathway and signalling through the EGF receptor (epidermal growth factor).<sup>2,5</sup> If Stat is removed, the germarium changes its shape and the germ line stem cells disappear; if, on the other hand, Stat is over-expressed in the shield cells, their number increases and there is intense proliferation of germ line stem cells. Therefore, the female germ line stem cells, apart from the signals mediated by BMP, need an extra signal released by the somatic shield cells, which in turn, need reverse control from the germ line stem cells, presumably mediated through the EGF receptor. Indeed, it seems that signalling via this receptor is essential to prevent an unlimited proliferation of germ line stem cells or of their progeny once they leave the niche. Indeed, the loss of function of the EGF receptor or of its Raf mediator causes an uncontrolled proliferation of early germ line cells (derived from primordial germ line cells). On the other hand, a ligand secreted by male early germ line stem cells, presumably Spitz, after to be processed by Rhomboid, a transmembrane protease, seems to activate the signalling via the EGF receptor in the cyst somatic cells.

Another protein involved in the control of niches in *Drosophila* is Notch. The ectopic activation of Notch in the somatic cells of the ovarian niche, other than the cap cells, induces an increase in the size of the niche and the formation of ectopic niches capable of maintaining and renewing the germ line stem cells. If, on the contrary, the signal through Notch is blocked, fewer cap cells are formed and the niches are smaller.

Interestingly, the niche of the germline of *Caenorhabditis* is also regulated through Notch.<sup>6</sup> Here the single niche cell expresses the Notch ligand LAG-2 (homologue of Delta) which activates GLP1 (homologue of Notch) in the germ line stem cells, controlling their self-renewal through the activation of Pumilio-like FBF genes which, in turn, repress the genes that promote meiosis. In this case, the orientation of the spindle in the germ line stem cells undergoing cell division seems to be irrelevant to their maintenance.

There are also different mechanisms used to replace germ line stem cells within the niches.<sup>4</sup> In the ovary of *Drosophila*, new germ line stem cells are generated by division oriented in parallel to the cap cells, resulting in two daughter cells that remain in the niche as stem cells, a process which has not been demonstrated in the male gonad. In addition, new germ line stem cells can be produced by reversion of transitory cells coming from 8 cell cysts, a mechanism that may be also occurring in males.

### The Mammalian Niches

The degree of complexity of the niches of vertebrates (mainly studied in mammals) is considerably higher than those described in invertebrates. However, as we mentioned earlier, while the evidence is less conclusive, many molecules involved in the control of niches in *Drosophila* and/or *Caenorhabditis* also play a role in these more evolved niches.

## The Niches of the Central Nervous System

In the adult central nervous system, the neurogenic niches are located in specialised areas: The subventricular zone of the lateral ventricles, which produce neurons that migrate to the olfactory bulb, and the subgranular zone of the dentate gyrus, that produce hippocampal neurons. In the case of humans, neurogenesis occurs in the subcortical white matter. Here the niches also need to provide the signals that, at least in part, determine neurogenesis. Subventricular zones homotypically transplanted produce many olfactory bulb neurons and glial progenitors grafted in subventricular zones turn into neurogenic progenitor cells.<sup>7-9</sup>

The main elements of the neurogenic niches are the ependymal cells, the blood vessels, and especially the glial cells given that, in addition to working as neural progenitors, they participate in the creation of the niches that regulate them. In fact, astrocytes are in contact with all the cell types known to be in the ventricular zone, including those of the intermediate transitional compartment and neural progenitors. Moreover, progenitors of the subventricular zone cocultured on astrocyte monolayers form colonies of neuroblasts, and soluble factors produced by astrocytes or expressed on their plasma membrane promote neurogenesis in cells of the subventricular zone. However, not all astrocytes have the capacity to generate neuroblasts (for example, those present in the spinal cord lack this ability), which is a reflection of the existence of specific neurogenic zones in the central nervous system. Indeed, as previously mentioned, some astrocytes work as neural stem cells while others have their role as the stem cells of the niche. On the other hand, the conditions of the astrocyte environment can determine the type of behaviour. Specifically, astrocytes which are differentiated in the presence of BMP or of LIF (leukaemia inhibitory factor) generate different types of cells: Those differentiated in the presence of BMP generate star-shaped cells, that are out-of-cycle and have limited neural stem cell potential, while, those differentiated in the presence of LIF produce bipolar or tripolar cycling cells, that express markers of neural progenitors and behave as such *in vitro*. Consequently, the ratio between BMP and LIF signalling may determine whether adult GFAP<sup>+</sup> cells of the subventricular areas or the dentate gyrus behave as niche cells or as neural stem cells.

There are many factors that seem to have a role in neurogenesis, and, although the results are not conclusive, many of them are similar to those described previously in the niches of *Drosophila* and *Caenorhabditis*. The most important are: EGF; FGF-2,<sup>10</sup> Notch 1 and its ligands,<sup>11,12</sup> Wnt;<sup>13</sup> morphogens, such as Shh, BMP and their antagonists;<sup>14,15</sup> and adhesion molecules, involved in cell-cell and cell-extracellular matrix interactions.<sup>8,9</sup>

EGF and FGF-2 seem to be the main mitogens involved in the *in vitro* expansion of neural stem cells, their effects being mediated by tenascin, which regulates the expression of the EGF receptor in these cells. Astrocytes in culture express FGF-2 and EGF and its receptors do it *in situ* in the neurogenic subventricular zone. In addition, it has been observed that there is a decrease in neurogenesis in the subventricular zone in rats deficient in FGF-2 or TNF $\alpha$ . Finally, a reduction of up to 60% in neurogenesis in the dentate gyrus has been reported in cystatin C-deficient mice, an autocrine/paracrine factor isolated from cultures of adult dentate gyrus and embryonic subventricular zones which enhances FGF-2 signalling.

Notch 1 and its ligands are also important for the control of self-renewal and differentiation of neural progenitors. In the subventricular zone, Jagged-1 and Notch 1 are expressed in closed groups of astrocytes, but do not ever seem to be co-expressed. *In vivo*, Jagged 1 promotes the self-regulation of the neural stem cells in the subventricular

zone, increasing their neurogenic potential. An increase of the number of Jagged-1<sup>+</sup>/Delta-1<sup>+</sup> neuroblasts activates Notch 1 in neural stem cells, increasing their self-renewal and blocking their differentiation, which results in the appearance of large numbers of astrocytes in the subventricular zones and in the inhibition of neuronal migration from these zones to the olfactory bulb.

Wnt is a family of secreted proteins that has many members and receptors as well as three different signalling pathways involving  $\beta$ -catenin, calcium and c-Jun. Various different Wnts are expressed *in vitro* in astrocytes derived from dentate gyrus and they have effect on neurogenesis. Wnt 3a and Wnt 5a promote *in vitro* proliferation of precursors and neuronal differentiation and, *in vivo*, the over-expression of Wnt 3 stimulates neurogenesis in the dentate gyrus while its inhibition blocks it.

As in other systems where the microenvironments regulate the biology of stem cells, Wnt and Notch seem to interact in the control of neurogenesis. For example, Wnt induces the expression of Jagged, a Notch 1 ligand.

Finally, several morphogens are also involved in adult neurogenesis in the central nervous system.<sup>14,15</sup> Cells that express Gli-1, one of the transcription factors involved in the signalling pathway of Shh (Sonic hedgehog), identified in the subventricular zone and dentate gyrus behave *in situ* as neural stem cells, and the over-expression of Shh in the dentate gyrus increases cell proliferation and neurogenesis, while cyclopamine, which inhibits Shh signalling, has the opposite effect. Moreover, Smoothened-deficient embryonic neural precursors (E 12.5), one of the subunits of Sonic receptor, also exhibit a decreased proliferation and neurogenic capacity. By contrast, another morphogen, BMP induces differentiation of subventricular zone progenitors into astrocytes. The subventricular zone produces BMP, its cells express receptors for these morphogens and their over-expression suppresses neurogenesis, while in neural cells it induces withdrawal from the cycle and the loss of the expression of typical markers of progenitors. Logically, BMP antagonists, such as Noggin and Ng1 (Neurogenesin-1) have the opposite effects. Noggin, like BMP, is expressed in neurogenic zones, especially in the ependyma, while Ng1 is expressed in astrocytes. Both BMP antagonists promote neurogenesis and inhibit gliogenesis. In addition, antisense RNA reduces the proliferation in the dentate gyrus by 40%, while the Noggin over-expression increases the expression of specific markers for progenitors and their proliferation. Presumably, in the neurogenic zones, the balance of the various morphogens (Shh, BMPs, Noggin) determines the behaviour of progenitor cells, which may change in the course of development, as demonstrated by the fact that, at some stages, BMP can induce neuronal formation instead of producing glial cells.

### **The Haematopoietic Niches of Bone Marrow**

The haematopoietic niches of bone marrow have been thoroughly investigated for several decades, although only recently the molecular basis of the interactions between haematopoietic stem cells and the components of bone marrow microenvironment starts to be understood. Moreover, these studies have generated many questions concerning previous interpretations on the cellular and histological organization of bone marrow.<sup>16-22</sup>

The “classical” model of haematopoiesis considered that the bone marrow showed a regionalized organization with the most primitive haematopoietic stem cells (HSCs) close to the endosteum and the developing blood cells forming a topological gradient of increasing maturation towards the blood sinusoids from where mature blood cells migrate to the bloodstream. This model has been confirmed by recent data demonstrating a direct



relationship between the number of osteoblasts in the endosteum and the number of HSCs (see below). However, the use of new markers to identify HSCs has questioned this model suggesting that there are, at least, two different microenvironments in the bone marrow. The first one, close to the endosteum, is where dormant/quiescent  $G_0$  HSCs would be stored. They would only start to divide at critical moments when there was urgent and massive demand for blood cells. However, true haemostasis of haematopoietic stem cells would occur in the currently termed vascular niche, where HSCs able to divide in response to the physiological demand for blood cells, that would be near the sinusoid blood vessels in association with reticular and/or endothelial cells.<sup>20,21,23</sup>

Other model based on kinetic results about the level of division of different haematopoietic progenitor cells points out that HSCs consist of three different subpopulations: Quiescent, homeostatic and activated.<sup>24</sup>  $CD34^- CD48^- CD150^{hi}$  LSK haematopoietic stem cells contain quiescent cells (about 30% of the total cell population) that divide every 150 days whereas the other 70% represent the named homeostatic HSCs dividing every 28-36 days. Therefore, quiescent  $CD34^-$  LSK progenitor cells could divide only 5 times during the normal adult mouse life span making impossible that this population significantly contributes to the daily blood cell homeostasis. On the contrary, no dormant, homeostatic HSCs would be responsible of the daily blood cell production. Dormant HSCs would be functionally activated only after stress or injury. These authors propose that the endosteal niches contain largely both quiescent and homeostatic HSCs whereas the perivascular niches might contain principally activated HSCs and their progeny. Thus, a flux of homeostatic HSCs migrate daily from the endosteal niches through perivascular niches to the circulation.

The finding of this new haematopoietic region, the perivascular niche, in the bone marrow was based on the use of new markers to identify haematopoietic stem cells ( $CD150^+ CD244^- CD48^- CD41^-$ ). These markers are members of the SLAM family, previously known as costimulatory molecules expressed by activated lymphocytes, and are mainly located close to the vascular endothelium, although there are cells with this phenotype also in the endosteal niche. Other results that support the existence of this vascular niche are: The fact that after mobilisation of haematopoietic stem cells out of the bone marrow using various treatments, such as cyclophosphamide or G-CSF, the cells moved near to splenic sinusoids; also, restored thrombopoiesis in thrombocytopenic mice (mice deficient in thrombopoietin or in its receptor c-Mpl) receiving CXCL12 and FGF-4 only occurred in the presence of bone marrow endothelial cells. Finally, the interaction of bone marrow endothelial cells, but not of those coming from vessels of nonhaematopoietic organs, with haematopoietic stem cells induces their proliferation.

Nonetheless, many of these conclusions have been questioned for several reasons: We do not know the true nature of the cell components of the bone marrow *in situ*, nor their potential functions *in vivo*; moreover, the methods used to identify *in situ* haematopoietic stem cells may not be the most appropriate. At first, it was considered that the low uptake of bromodeoxyuridine by haematopoietic cells close to the endosteum was attributed to them being out-of-cycle stem cells or having very long cycles, given their low rate of renewal in homeostatic conditions; however, better tests have confirmed that there really was less uptake of the marker in the region of the bone marrow compared to other areas. Further,  $CD150$  cannot be considered a bona fide specific marker exclusive to haematopoietic stem cells in all species. On the other hand, the deletion of many molecules that presumably participate in the interactions between haematopoietic stem cells and the stromal cells, which have been considered to be a key tool to postulate their



function in these processes, has not been in most cases, carried out selectively in each of the cells involved, which may have lead to some significant errors.

In any case, the relationship between bone (osteoblasts) and haematopoietic stem cells, without doubt, has been the greatest focus of attention in recent years. The first demonstration of this relationship, although indirect, is their simultaneous appearance during the development of the bone and haematopoietic niches. In embryonic mice, the first mesenchymal condensations that will give rise to bones occurs at day 12.5 of gestation; around the day 15, the process of calcification of chondroblasts starts leading to the appearance of the first osteoblasts. Between day 15.5 and day 17.5 there is vascular invasion that facilitates the formation of bone, organizes the bone marrow and enables the first haematopoietic stem cells to be “seeded”. Further, recent results<sup>25</sup> also suggest that endochondral ossification is important to organize the haematopoietic niches. CD105<sup>+</sup> Thy-1<sup>+</sup> cells isolated from E14.5 mouse bone and grafted under the kidney capsule recruit host blood vessels, produce an ectopic bone after undergoing endochondral ossification and generate a marrow cavity that houses long-term HSCs. On the contrary, CD105<sup>+</sup> Thy-1<sup>+</sup> cells are able to produce bone but not a true haematopoietic bone marrow. Similar results had been previously reported by other authors.<sup>26</sup> Therefore, it seems necessary the existence of a suitable bone microenvironment for that the haematopoietic stem cells are stored in the bone marrow, although they already exist previously in the foetal liver.

An objection to this interpretation is that haematopoiesis occurs in all vertebrates, but haematopoietically active bone marrow is a relatively recent “phylogenetic invention” that occurs in the most advanced urodele amphibians and most primitive anurans. In other amphibians and in all fishes, haematopoiesis occurs in niches which lack osteoblasts but contain other key elements characteristic of the bone marrow microenvironment, such as reticular cells and vascular sinusoids.<sup>27</sup>

It is true, however, that when HSCs are intravascularly administered, they rapidly disappear from the bloodstream to be stored in the endosteal stem cell niches. Further, more conclusive studies in recent years have demonstrated that an increase in the number of osteoblasts leads to an increase of the number of haematopoietic stem cells and viceversa. Specifically, a decrease in the signalling transmitted by BMPRIa, a BMP receptor, and the constitutive expression of the protein related to the parathyroid hormone that increase the number of osteoblasts also produce an increase in haematopoietic stem cells.<sup>17,19</sup> Some of the mechanisms underlying this relationship between osteoblasts and haematopoietic stem cells have been identified, and, in particular, the concentration of Ca<sup>++</sup> in the endosteum seems to play a key role. Seemingly, haematopoietic stem cells “recognise” the concentration of Ca<sup>++</sup> through the CASR receptor (Ca<sup>++</sup> sensing receptor) which they express. It is known, on the other hand, that changes in the concentration of Ca<sup>++</sup> affect the equilibrium between bone production and destruction in which osteoblasts and osteoclasts participate. In CASR-deficient mice, haematopoietic stem cells, which are CXCR4<sup>+</sup>, migrate in response to the chemokine CXCL12 (or SDF-1) to the endosteum but are unable to anchor there.

However, various recent reports contradict some of these results and question the tight relationship between endosteal osteoblasts and haematopoietic stem cells.<sup>21</sup> Firstly, it should be taken into account that on the surface of the endosteum, not all the cells are osteoblasts, but there are also osteoclasts, reticular cells and blood vessels, with which logically, should be taken into account to determine the real/true organisation of the endosteum niche. Also, apart from osteoblasts, some of these cells are capable of secreting molecules involved in the interactions with haematopoietic stem cells. For example, reticular

cells are capable of secreting CXCL12 which attracts stem cells and megakaryocytes and perivascular cells secrete angiopoietin, another molecule involved in these processes (see below). Further, “biglycan” mice which are deficient in osteoblasts have normal numbers of haematopoietic stem cells.

Another of the most striking results is that of a study that reports G-CSF, a growth factor that releases haematopoietic stem cells to the bloodstream, apparently having an effect on osteoblasts.<sup>28</sup> Such effect seems to depend on direct or indirect stimulation of noradrenalin release that would reduce the activity of osteoblasts, and consequently, the levels of CXCL12, which would mobilise stem cells from the endosteum. More recently, the same research group demonstrated that the migration of bone marrow stem cells and the levels of CXCL12 are subject to circadian rhythms mediated, at least in part, by the levels of noradrenalin themselves.<sup>29</sup> These studies also suggest that osteoblasts are not the direct “target” of noradrenalin. In fact, this hormone activates  $\beta_3$  adrenergic receptors and osteoblasts express  $\beta_2$  receptors. Rather, adrenergic signals are locally delivered by nerves in the bone marrow and transmitted to stromal cells by  $\beta_3$  adrenergic receptors.<sup>29</sup> Other catecholamines as well as tachykinins and other neurotransmitters affect also the haematopoietic cell mobilization.<sup>30,31</sup> Dopamine stimulates the GM-CSF dependent mobilization of CD34<sup>+</sup> haematopoietic progenitors via D3 and D5 receptors.<sup>30</sup> The bone marrow receives innervations by substance P-secreting neurons and substance P is related to the SCF/c-kit pair, which has trophic effects on haematopoiesis, and increases the GM-CSF production. On the other hand, the above mentioned role of CXCL12 in haematopoiesis could be secondary to the induction of *TAC1* gene which encodes substance P, neurokinin A (NKA) and their extended forms.<sup>31</sup>

Notch and its ligands, and some molecules of the Wnt family have also been shown to be involved in the control of the haematopoietic niche, although the results are, once again, contradictory.<sup>17,19</sup> Osteoblasts express Jagged-1, a Notch ligand that transmits signals through Notch to haematopoietic stem cells, but they stop doing so when the cells differentiate. Some results indicate that overactivation of Notch in haematopoietic stem cells increases their capacity for self-renewal, and directs their differentiation toward the lymphoid lineage, while other studies have not found any effect on the renewal capacity of such cells after inactivation of Notch in haematopoietic stem cells or Jagged-1 in the haematopoietic stroma. Also haematopoietic stem cells isolated from Notch-deficient mice normally repopulate irradiated Jagged-deficient mice, even in the presence of stem cells coming from wild-type mice.

The findings concerning the role of Wnt in haematopoiesis remain unclear. Like the Notch one, the Wnt signalling pathway is active in haematopoietic stem cells, but there is no agreement on its role. Some results demonstrate that Wnt 3a and the  $\beta$ -catenin overexpression, involved in the canonical Wnt signalling pathway, promote self-renewal of HSCs, while the blocking of the signalling stops their ability to re-establish haematopoiesis in irradiated mice and mice that constitutively express different active forms of  $\beta$ -catenin show alterations in haematopoiesis. However, other studies have found no such defects in the absence of  $\beta$ -catenin and indicated that haematopoietic stem cells deficient in this molecule showed a normal rate of self-renewal.

Adhesion molecules and components of the extracellular matrix of the bone marrow are also important, not only for anchoring the haematopoietic cells to the bone marrow stroma, but also to regulate their survival, proliferation and differentiation, it having been concluded that adhesion to the surface of the endosteum is essential to maintain haematopoietic cells quiescent. The pair formed by angiopoietin, a molecule secreted

by osteoblasts, and Tie2/Tek (Endothelial-specific receptor tyrosine kinase), its receptor expressed in the haematopoietic stem cells, promotes this adherence, quiescence and survival. Also osteopontin, an anchor for integrins and CD44 expressed in stem cells, is a regulator of the size of stem cell pool. In mice deficient in this molecule, there is an increase in the number of haematopoietic stem cells and a decrease in their death in relation to an increased expression of Tie2 and Jagged-1. Here, as in other niches, the cadherin-mediated interactions are also important. Homotypic interactions between N-cadherin molecules are involved in the regulation of the anchoring of haematopoietic stem cells to the endosteum, but also in their self-renewal and differentiation. N-cadherin is a target of the action of angiopoietin 1 and regulates the cell cycle; accordingly, in mice whose haematopoietic stem cells are deficient in myc oncogene, and that show a dramatic expansion and an inhibited differentiation of haematopoietic progenitors, it has been reported that there is an increase of the expression of N-cadherin and integrins. However, other studies have concluded that N-cadherin deficiency does not affect the number of haematopoietic stem cells or haematopoiesis in general, concluding that N-cadherin-expressing cells close to the endosteum may not have characteristics of stem cells, and, moreover, that cells with such characteristics do not express N-cadherin.<sup>21</sup> Further, changes in the pattern of expression of the adhesion molecules in the bone marrow alter the localisation and behaviour of haematopoietic stem cells. For example, metalloprotease 9, which degrades osteopontin, reduces the anchoring of stem cells, inducing them to migrate and enter to the cell cycle; also the activity of such metalloprotease and of other enzymes that degrade the extracellular matrix is regulated by the activity of the osteoclasts via RANKL.

A particularly important aspect to address some of the questions with regards to haematopoietic niches is our understanding of the nature of the cells of these niches. Bone marrow is histologically organised into a network of reticular cells that houses developing haematopoietic cells as well as adventitial reticular cell, that are associated to the sinusoidal walls. However, these data are not sufficient to enable us to ascertain the potential of the stroma or its relationship with the haematopoietic stem cells. At present, it is considered that CXCL12-rich cells are a key element in haematopoietic niches<sup>21,23</sup> but their relationship with reticular and adventitial reticular cells is not clear. Are all the cells equal, or do they determine different “environments” in the endosteum, vascular niche and reticular network?. How are these “environments” related? Many of these questions have not been solved with the isolation of nonhaematopoietic cells from the bone marrow, which seem to have characteristics of stem cells, and even in some cases give rise to haematopoietic stem cells, but whose relationships with the elements of the stroma are also far from clear.<sup>32</sup> Among all of these cells, the most interesting are the so-called mesenchymal stem cells or multipotent mesenchymal stromal cells, which some authors consider to be the *in vitro* equivalent of the adventitial reticular cells described *in situ*. Mesenchymal cells are derived from heterogeneous colonies of bone marrow fibroblasts, that, in certain occasions, accumulate Ca and fat, and undergo osteogenic differentiation, and to a lesser extent adipogenic and chondrogenic differentiation.<sup>32</sup> Despite their possible relationships with haematopoietic stroma, mesenchymal cells are most abundant in bone and, in general, can be isolated from all types of connective tissue. They do not express haematopoietic markers (CD14, CD34, CD45) but they express adhesion molecules (CD73, CD90, CD105, CD166) and other markers (CD49a-f, CD51, CD106, CD133, CD271, Stro-1, SSEA 1, 4, 3G5), including receptors for many cytokines (IL1, IL3, IL4, IL6, IL7, IL15, IFN $\gamma$ , TNF $\alpha$ ) and chemokines (CCR1, CCR7, CXCR4,

CXCR5, CXCR6). One of the most striking characteristics of mesenchymal cells, which have become an important source of stem cell for certain clinical trials, is that they have immunosuppressive properties, although the factors involved in this immunosuppression and their specificity remain unknown.

A PDGFR $\alpha$ <sup>+</sup> Sca-1<sup>+</sup>CD45<sup>-</sup>TER119<sup>-</sup> mesenchymal stem cell population has been isolated from adult mouse bone marrow.<sup>33</sup> This population in vivo transplanted mainly differentiated into osteoblasts and Angiopoietin 1 and/or CXCL12-expressing perivascular cells. However, presumptive descendants of this population that downregulate Sca-1 produce 10 times more CXCL12 than their Sca-1<sup>+</sup> counterparts.<sup>33</sup> Previously, other authors identified a stromal progenitor in human bone marrow that highly expresses MCAM (melanoma-associated cell adhesion molecule)/CD146, regenerates bone and stroma and establishes the haematopoietic microenvironment in vivo.<sup>26</sup> This molecule is expressed by subendothelial cells of bone marrow sinusoids in situ and grafted human CD146<sup>+</sup> cells give rise in vitro to CD146<sup>+</sup> subendothelial cells in the in vivo formed bone marrow. In addition, ex vivo CD146<sup>hi</sup> CFU-Fs exhibit the same phenotype of adventitial reticular cells which in situ reside close to the endothelial walls. On the other hand, sorted CD146<sup>+</sup> Ng2<sup>+</sup> CD34<sup>-</sup> CD45<sup>-</sup> CD56<sup>-</sup> pericytes, a mesenchyme-derived cell-type, from numerous tissues are able to produce under adequate cultured conditions myocytes, chondrocytes, adipose cells and osteocytes, even in clonal conditions.<sup>34</sup> Furthermore, in situ CD146<sup>hi</sup> pericytes express typical markers of mesenchymal stem cells such as CD44, CD73, CD90 and CD150 and a CD146<sup>+</sup> subendothelial cell population produces bone and marrow after subcutaneous grafting in SCID mice.

Taken together all these results we can conclude that the bone marrow stroma includes CD146<sup>+</sup> progenitor cells that presumably represent in situ adventitial reticular cells that express different amount of CXCL12 and angiopoietin 1, and in vitro the so-called mesenchymal stem cells.

Adipocytes are other major cell component of bone marrow microenvironment of bone marrow that also seem affect the behaviour of haematopoietic stem cells. Bone marrow rich in adipocytes contain low numbers of HSCs and A-ZIP/F1 mice that exhibit a deficient adipogenesis show a massive osteogenesis and accelerated recovery of haematopoiesis.<sup>35</sup> Negative effects of adipocytes on hematopoiesis are mediated, at least in part, by neuropilin 1, a coreceptor to a tyrosine kinase receptor.<sup>36</sup>

Before finishing this section, it should be noted that the importance of haemato-poietic niches for the control of haematopoiesis is illustrated, in physiological conditions during the ontogeny and phylogeny of vertebrates. During the ontogeny in mice and humans, haematopoiesis occurs in various different organs: Primitive streak, vitelline sac, AGM region, placenta/allantoid, foetal liver and, finally, the bone marrow, each of them with haematopoietic capacity, and presumably with different niches, though this has been little explored to date.<sup>37</sup> Also, many lower vertebrates, such as lampreys and amphibians, that undergo dramatic metamorphosis, modify their blood cell-producing organs in relation to the anatomical and histological changes that occur in their microenvironments.<sup>27</sup>

## HOMEOSTASIS IN THE NICHES

As we said at the beginning, the relevance of microenvironments for the control of the biology of the stem cells is already beyond doubt, but research has raised more questions

in this field than it has answered. Some of the questions are obvious: How can the niches be refilled if they are emptied? Does this occur physiologically? Are the mechanisms involved the same in all systems and under all conditions?

It is clear that there is homeostasis in the niches at a level which varies across species and situations, with different, though not mutually incompatible, strategies having been developed to solve the problem. So, new niches can be created, which is indeed evident in the case of haematopoietic niches in relation to the constant remodelling of bone. At the same time, it seems that it is possible to recover an empty niche by symmetric proliferation of the adjacent cells.

What is proliferation rate of stem cells within the niches? Presumably this is different in different conditions. During the development of some invertebrate models, stem cells slowly divide per se, until the niche is filled, and any excess cells leave the niche and start to differentiate. In other cases, as we have seen, only in the presence of signals released by the niche do the stem cells proliferate. It seems logical that the first model occurs during development and the second in adult niches.

Do niche cells age or undertake pathological changes? Can these changes affect the behaviour of the stem cells that they home? In old *Drosophila*, homeostatic control of gonadal niches is less effective and the number of germinal stem cells decreases probably due to the loss of adhesion with the cells of the niche, which is mediated, as described previously, by E-cadherin or a decreased Jak-Stat signalling.<sup>2,3</sup> Interestingly, the loss of germinal stem cells may be reverted through the over-expression of the superoxide dismutase gene which reduces damage, induced by the free radicals released by the process of ageing, to the stem cells and/or the cells of the niche (2). Aged niches inhibit, both in vivo and in vitro, the expression of stem cell markers such as Oct4 in embryonic stem cells and Myf-5, in muscle satellite cells, and reduce myogenic proliferation and differentiation in both these stem cell lineages, although the effect can be antagonized in embryonic cells but not in adult satellite cells.<sup>38</sup> Recently, Mayack et al<sup>39</sup> using complementary in vitro and in vivo heterochronic models, demonstrated that age-associated changes in the niche cells deregulated normal haematopoiesis, a defect that could be reversed by exposure to a “young” circulation or by neutralization of IGF-1 in the marrow microenvironment.

Obviously, like normal cells, neoplastic cells depend, at least in part, on their environment. For example, neurofibrin-deficient Schwann cell tumours are favoured when they develop in environments that are heterozygotic for this molecule while when they grow in normal environments, the growth is less tumorigenic. Also the presence of mesenchymal stem cells near or in mammary tumours increased the metastatic capacity of the tumour cells.<sup>3</sup>

Recent results that show the development of myeloproliferative syndromes as a consequence of mutations which only affect the microenvironment of the bone marrow but not the haematopoietic stem cells themselves, or that they affect the both compartments, are even more interesting.<sup>3,40</sup> Specifically, the removal of retinoblastoma protein, a regulator of the cell cycle and tumour inhibitor, generated extramedullary haematopoiesis and a myeloproliferative syndrome. The syndrome only appeared when the protein disappears from the microenvironment. It can then be concluded that the disease is consequence of the interaction between myeloid cells and the altered environment. On the other hand, retinoic acid receptor  $\gamma$  deficiency in the bone marrow environment also causes a myeloproliferative syndrome, although in this case there is no mobilization of haematopoietic stem cells.

## CONCLUSION

There are very many questions, and so far few answers, but the importance of microenvironments for the control of stem cells is now unquestionable. This realisation opens the possibility of new approaches based on an understanding of the biology of these cells, which will enable, without doubt, more effective therapeutic use of stem cells. A better understanding of the cellular components of microenvironments and their cellular and molecular interactions with the other components of the niche, including the stem cells themselves, will be key to make progress in this field.

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## CHAPTER 11

# MOBILIZATION AND HOMING OF HEMATOPOIETIC STEM CELLS

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**Abstract:** Hematopoietic stem cells (HSC) are a population of precursor cells that possess the capacity for self-renewal and multilineage differentiation. In the bone marrow (BM), HSCs warrant blood cell homeostasis, but at the same time a stable pool of functional cells must be constantly maintained. For this, HSCs constitute a model in which subpopulations of quiescent and active adult stem cells co-exist in the same tissue, in specific microenvironment called stem-cell "niches." These microenvironments keep the stem cells at quiescent (osteoblastic niche) for its self-renewal and activate the stem cells (vascular niche) for proliferation and/or injury repair, maintaining a dynamic balance between self-renewal and differentiation. HSC reside in the bone marrow but can be forced into the blood, a process termed mobilization used clinically to harvest large number of cells for transplantation. At the same time, homing to the BM is necessary to optimize cell engraftment. Here, we summarize current understanding of HSC niche characteristics, and the physiological and pathological mechanisms that guide HSC mobilization both within the BM and to distant niches in the periphery. Mobilization and Homing are mirror process depending on an interplay between chemokines, chemokine receptors, intracellular signaling, adhesion molecules and proteases. The interaction between SDF-1/CXCL12 and its receptor CXCR4 is critical to retain HSCs within the bone marrow. Current mobilization strategies used in clinic, mainly G-CSF cytokine, are well tolerated but often produce suboptimal number of collected HSCs. Novel agents (AMD3100, stem cell factor, GROβT.) are being developed to enhance the mobilization to modify the signaling into the niche and boost the stem cell harvest, increasing the number of HSCs available for the transplant.

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## INTRODUCTION

Over half a century after scientists realized that all blood cells are derived from one stem cell, Tim and McCulloch<sup>1</sup> defined the basic concept of stem cells; cells with the capacity for self-renewal (duplication without losing development potential) and the potential to give rise to multiple cell types (differentiation). Three decades later, Hematopoietic Stem Cells (HSC) were definitively identified and isolated in the Thy-1<sup>lo</sup> Lin<sup>-</sup> Sca-1<sup>+</sup> fraction.<sup>2</sup> All mature blood cells, which have a limited life span, are continuously produced throughout the life of the organism by a highly hierarchical process called hematopoiesis, but at the same time a stable pool of functional HSCs must be constantly maintained. For this, HSC constitute a model in which subpopulations of quiescent and active adult stem cells co-exist in the same tissue.

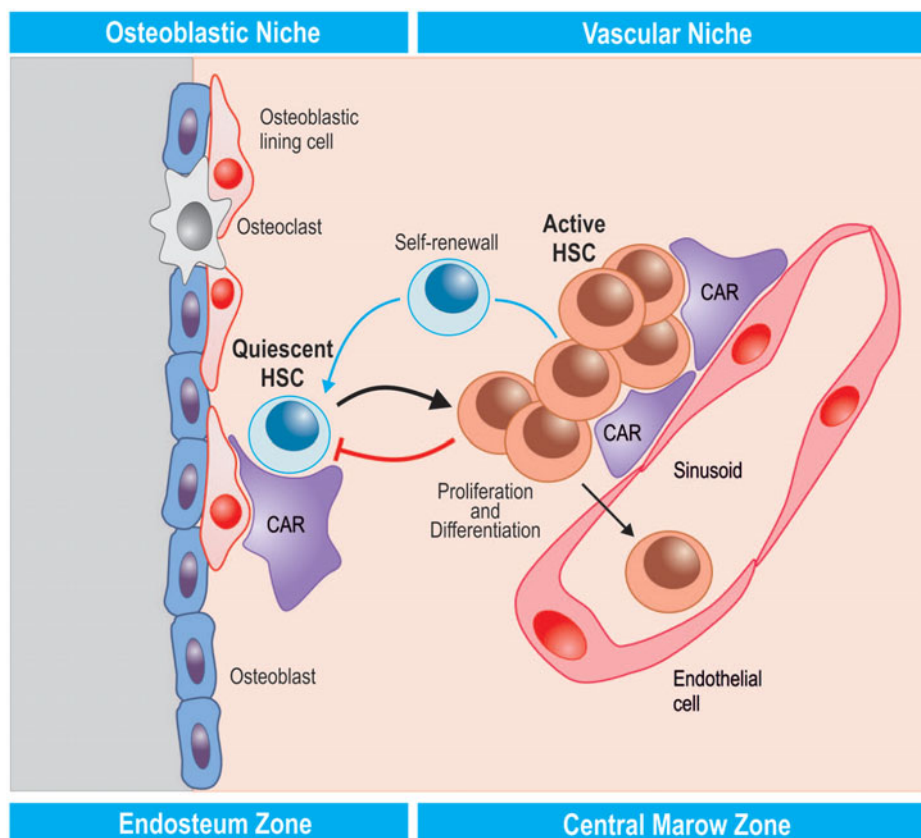
During the homeostasis, the majority of HSC reside in specific microenvironment called stem-cell “niches” localized in the bone marrow. These microenvironments keep the stem cells at quiescent (osteoblastic niche) for its self-renewal and activate the stem cells (vascular niche) for proliferation and/or injury repair, maintaining a dynamic balance between self-renewal and differentiation.

In this chapter, we focus on recent findings regarding the location, structure and molecular activities of the HSC microenvironments and the processes which regulate HSC mobilization from bone marrow to secondary organ sites and back (homing) to the bone marrow compartment. The knowledge about the cellular niche components and the signaling molecules between HSC and niche cells will be necessary for the successful expansion of HSC for therapeutic use. Therefore, we will review the new pharmacological agents used to modify the signaling into the niche and boost the stem cell harvest.

## HSC MAINTENANCE

Hematopoietic Stem Cells (HSCs) constitute a model in which two subpopulations, one being long-term quiescent (“dormant”) and the other being more actively cycling (“primed”), co-exist in the same tissue.<sup>3</sup> Primed HSCs are the reservoir which supports the daily production of billions of blood cells, whereas quiescent HSCs function as a backup or reserved subpopulation. This reserved population can be activated either by a stochastic mechanism or by feedback upon loss of active stem cells or extensive tissue damage.<sup>4</sup>

One of the main questions is how can these two subpopulations be maintained in the same locations. For this, an alternative “zoned” model had been proposed in which there are segregated zones that determine the active or quiescent state of the stem cells (Fig. 1). In 1978, Schofield<sup>5</sup> first put forth the HSC “niche” concept to describe the physiological microenvironment in which HSCs reside. These niches are specialized microenvironments within bone marrow created by supporting cells that express membrane-bound and secreted factors that promote HSC maintenance (survival and self-renewal),<sup>6</sup> and that regulate HSC migration and differentiation. Within the bone marrow, some HSCs are found close to the endosteal lining in the cavities of trabecular bone. These niches, termed endosteal or osteoblastic niches contain quiescent and homeostatic HSCs, and maintains the balance between quiescent HSC and trans-marrow migration. By contrast, perivascular niches are located more centrally in bone marrow cavities and contain sinusoidal endothelial cells, reticular cells and macrophages, being essential to support injury-activated HSCs and their immediate progeny.



**Figure 1.** Proposed “zoned” model for Hematopoietic stem cells. Quiescent hematopoietic stem cells (HSCs) are localized in the endosteal zone closed to osteoblastic lining cells. These cells together with endothelial, osteoblastic, osteoclast and CAR (CXC-chemokine ligand 12-abundant reticular) cells constitute the Osteoblastic Niche. Whilst active HSCs are situated in the central marrow region, stimulated by endothelial, megakaryocyte and CAR cells, lacking of osteoblastic cells and forming the Vascular Niche. In this zoned stem cell model, active stem cells are primed subpopulations that account for most of the replenishment of corresponding tissues, whereas quiescent stem cells function as a backup or reserved subpopulation. Therefore, quiescent HSCs replace damaged active stem cells (black arrow), and conversely, active HSCs may replace lost quiescent cells (blue arrow). A negative feedback from either active HSC or their progeny (red line) may contribute to prevent quiescent HSCs from activation..

### Osteoblastic Niche

Osteoblasts, osteoclasts and other mesenchymal-derived stromal cells, such as reticular cells, fibroblasts and adipocytes, create a supportive microenvironment for HSCs within the bone marrow, named endosteal or osteoblastic niche. Elegant imaging studies by Nilsson et al<sup>7</sup> showed that primitive HSCs reside close to the endosteal bone surface. When osteoblasts were experimentally manipulated,<sup>8,9</sup> a positive correlation between the number of osteoblasts and the number of HSCs was observed, leading to the hypothesis that osteoblasts were essential components of the HSC bone marrow niche.

The endosteum (inner surface of the bone) is covered by a protective layer of bone-lining cells which can differentiate into bone-forming osteoblasts; and by the presence of bone-resorbing osteoclasts which are important in stem cell mobilization.<sup>10</sup> Osteoblasts have been suggested to secrete factors that regulate HSC maintenance, including angiopoietin (ANG-1), thrombopoietin (THPO) and CXC-chemokine ligand 12 (CXCL12, also called SDF-1) which will be described in detail below. Functional studies indicate that ANG-1 binds to its receptor Tie-2 on HSCs inducing adhesion and quiescence within the niche, whereas CXCL12 regulates HSC migration and localization in the bone marrow. Additional studies had shown that osteoclasts secrete proteases such as matrix metalloproteinase 9 (MMP9) and cathepsin K, which are required for bone remodelling, as well as to release membrane-bound growth factors, such as stem-cell factor (SCF) that regulate HSC frequency and localization in the bone marrow. Perivascular cells could be critical sources of angiopoietin and CXCL12 for HSCs that reside at or near the endosteal surface and much of the thrombopoietin in the endosteal region may enter the bone marrow through the circulation.

Other fundamental question is whether HSC require cell-cell contact with supporting cells at the endosteal surface. One controversial study proposes that HSC adhere to the osteoblasts surface using N-cadherin-mediated homotypic adhesion and the HSCs are dependent upon this interaction for the maintenance. The most potent quiescent HSCs in the endosteum are maintained in a hypoxic environment to protect them from oxidative damage by reactive oxygen species.

In short, the interaction of bone cells, haematopoietic cells and vascular cells at the endosteum regulates both haematopoiesis and bone formation, showing the existence of an endosteal niche for HSCs.

### **Vascular Niche**

It is known that the vasculature also has a crucial role in the formation and expansion of HSC during embryonic developments and in the regulation of adult HSCs. In mammals, the ability of HSCs to self-renew and differentiate during fetal development, before the creation of bone-marrow cavities, suggests that HSCs are maintained in niches that do not involve bone. Adult HSCs are also present in extramedullary tissues, such as the liver and spleen, so extramedullary haematopoiesis can occur in these tissues for long periods of time, despite the absence of bone or endosteum.

When the localization of HSCs in bone marrow has been studied using signaling lymphocyte activation molecule (SLAM) family markers,<sup>11</sup> about 60% of bone-marrow HSCs were found adjacent to sinusoids and up to 20% of HSCs localized to the endosteum. Sinusoids are specialized blood vessels that are present in haematopoietic tissues and through which venous circulation occurs. The walls of sinusoids are composed of endothelial cells through which HSCs can enter and exit the circulation. Endothelial cells can promote the maintenance of HSC in culture, but one open question is whether endothelial or perivascular cells can create a perivascular niche that helps to maintain HSCs or whether HSCs transiently migrate through perivascular sites on their way in and out the circulation.

Similar to the endosteum, the perivascular environment also contains multiple cell types that may regulate HSCs. Sugiyama et al<sup>12</sup> found that perivascular reticular cells in bone marrow express very high levels of CXCL12, a factor required for the maintenance of HSCs. The presence of CXCL12-secreting reticular cells in both the perivascular and

endosteal environments provides a mechanism for the maintenance of HSCs in both sites, and reminds that the most morphological recognizable cell types in a particular environmental (such as osteoblasts and endothelial cells) might not be the cells that are most functionally important for the creation of the niche. Additionally, other cells localizing to the perivascular environment might have a role in the maintenance of HSCs, including megakaryocytes and mesenchymal progenitors which may also regulate hematopoiesis secreting factors such as angiopoietin and CXCL12. The localization of HSCs in the vascular niche enables rapid release into the circulation, which might be particularly important in pathology including ischemia and malignancy.

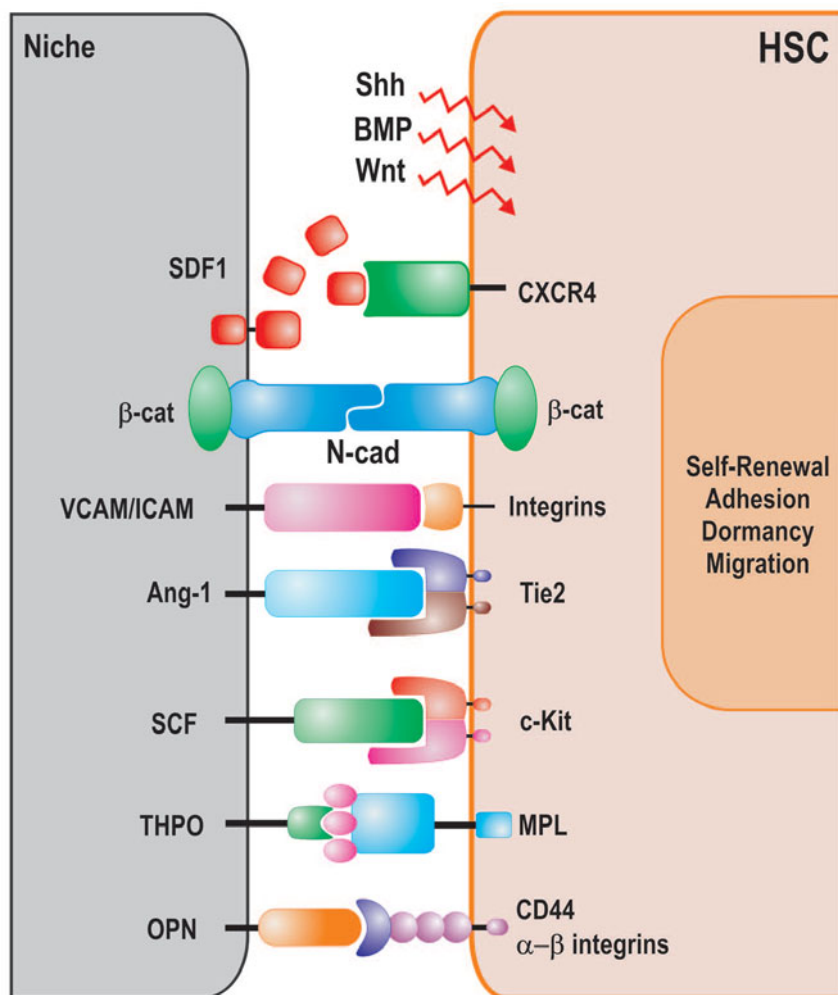
### HSC-Niche Interactions: Molecular Pathways

Much is known about the osteoblastic and vascular microenvironments but the interrelationships and interactions with HSCs in vivo remain largely unknown. Osteoblastic signals normally inhibit cell proliferation whilst in the central marrow are dominant the vascular signals that favor cell proliferation. A combination of chemokines, growth factors, adhesion molecules and signaling pathways are required to function in concert and maintain the HSCs in a state of quiescence or to be mobilized and recruited to the vascular niche (Table 1). Now, we revised some of these components that have been shown to be functionally involved in controlling HSC-niche interactions.

**Table 1.** Stem cell-niche interaction: Molecules involved in mobilization and homing

Niche Stroma Ligand	HSC Receptor	Function	Reference
N-Cadherin (under controversy)	N-Cadherin	Adhesion	13-15
Soluble Kit Ligand (SCF, stem cell factor)	c-Kit	Self-renewal, Mobilization	16
Angiopoietin (Ang-1)	Tie-2	Self-renewal, Adhesion	18
Stromal derived factor-1 (SDF-1)	CXC chemokine receptor-4 (CXCR4)	Self-renewal, Mobilization, Homing	19,37
Vascular cell adhesion molecules-1 (VCAM-1)	Very Late Antigen-4 (VLA-4)	Adhesion, Mobilization, Homing	20,44
ICAM-1, 2, 3	LFA-1	Adhesion, Mobilization, Homing	45
Thrombopoietin (THPO)	MPL	Self-renewal	22
Osteopontin (OPN)	$\alpha$ - and $\beta$ integrins, CD44	Adhesion	23
Endothelial (E)- selectin	E-selectin ligand-1 (ESL-1)	Adhesion, Homing	42
P- selectin	P-selectin glucoprotein ligand-1 (PSGL-1)	Adhesion, Homing	43

Quiescent HSCs are tightly anchored in endosteal niche through interactions with several adhesion molecules expressed by both HSCs and niche stromal cells (Fig. 2). Although still under controversy, it has been postulated that homotypic N-cadherin interactions play an important role in anchoring HSCs to the endosteal niche. An increased number of osteoblasts expressing N-cadherin were correlated with an increase in HSC number, suggesting that this molecule is a key component of the niche to support HSCs.<sup>13</sup>



**Figure 2.** Niche signaling. In order to maintain physiological homeostasis, the niche require a combination of cytokines, growth factors, ligands, adhesion molecules and signaling pathways necessary to achieve a delicate balance between HSCs self-renewal and differentiation. Several receptors that bind soluble or membrane-bound ligands produced by niche cells have been shown to be crucial for the maintenance of quiescent HSCs. Abbreviations: SDF1, stromal derived factor-1; CXCR-4, CXC-chemokine receptor-4; N-cad, N-cadherin; β-cat, β-catenin; VCAM, vascular cell-adhesion molecule; ICAM, intercellular adhesion molecule-4; Ang-1, angiopoietin-1; SCF, stem cell factor; THPO, thrombopoietin; OPN, osteopontin; Shh, Sonic hedgehog; BMP, bone marrow proteins; Wnt, Wingless.



However, two recent studies<sup>14,15</sup> of conditional knockout mice lacking N-cadherin specifically in the hematopoietic system has shown that N-cadherin is not necessary for niche function and proper hematopoiesis.

One of the first molecules identified as playing a role in survival of HSC were the Stem Cell Factor (SCF) and its receptor c-Kit. Analysis of the different SCF and c-Kit mutants revealed that although not essential for the generation and initial expansion of HSCs in the embryo and fetal liver, they are crucial for long-term maintenance and self-renewal of adult HSCs in the BM.<sup>16</sup> Membrane SCF (mbSCF) is expressed on osteoblasts and has a higher and more sustained capacity than soluble SCF to activate c-Kit, which is expressed at high levels by HSCs.<sup>17</sup> Angiopoietin-1 (Ang-1)-Tie 2 is an essential signaling for the maintenance of HSCs in a quiescent state. Ang-1 is expressed and secreted by osteoblasts, and interacts with the Tie2 tyrosine kinase receptor expressed on quiescent long-term HSCs, enhancing the ability of HSCs to become quiescent and induces their adhesion to bone.<sup>18</sup>

Additionally, HSC expresses CXCR4, the receptor of stromal-cell-derived factor 1 (SDF-1; also known as CXCL12) expressed by CAR cells, osteoblasts and vascular endothelial cells.<sup>19</sup> The CXCR4-SDF-1 interaction is an essential axis in the maintenance, retention and mobilization of HSCs during homeostasis and after the injury, so CXCL12-deficient embryos have severely reduced HSC numbers and function. Its expression and secretion is induced in response to HSC loss, due to irradiation, chemotherapy or hypoxia. Interactions through CXCL12 can lead to up-regulation of vascular cells adhesion molecule-1 (VCAM-1),<sup>20</sup> which has also been involved in the localization and retention of HSCs within niche. Activation of other adhesion molecules, such as very late antigen 4 (VLA-4) and leukocyte function antigen 1 (LFA-1) is also necessary for these movements. Integrin- $\alpha 4\beta 1$  (VLA-4) is involved in the mobilization and migration of HSCs or progenitor cells, and integrin- $\beta 2$  (LFA-1) has been used to discriminate long-term from short-term reconstituting HSCs and progenitors. Similar to SDF-1, fibroblast growth factor (FGF)-4 promote hematopoietic progenitor cells recruitment and adhesion to the vascular niche. Therefore, a gradient between vascular niche (high) and osteoblastic niche (low) has been proposed to be important for the recruitment of HSC.<sup>21</sup> Other growth factors such as vascular endothelial growth factor (VEGF) and placental growth factor (PLGF) are also involved in HSC mobilization and/or recruitment.

The cytokine thrombopoietin (THPO) has a double role, by one hand regulate the production and differentiation of megakaryocytes and platelets, and in other hand regulate HSC quiescence. HSCs lacking of the thrombopoietin receptor MPL,<sup>22</sup> are exhausted over time due to overproliferation and loss of quiescence. Similar to MPL mutants, inhibition of THPO in osteoblasts reduces the number of quiescent HSC, while exogenous THPO inhibit the proliferation. The acidic glycoprotein Osteopontin (OPN) is secreted by osteoblasts and might function as a negative regulator of HSCs pool size. It had been postulated that the increase of HSCs in OPN deficient mice is due to a decreased apoptosis.<sup>23</sup> Finally, critical components in the interaction of HSCs are the Rho GTPases, Rac1 and Rac2. Deletion of both genes in engraftment HSCs *in vivo* leads to massive mobilization of HSC to the peripheral blood.<sup>24</sup> Interestingly, Rac is activated by adhesion via  $\beta 1$ -integrin and via stimulation of CXCL12-CXCR4 and by CSF-c-Kit signaling.<sup>25</sup>

Recently, several signaling pathways have emerged as important control devices of HSC fate, including Notch, Wingless-type (Wnt), Sonic hedgehog (Shh) and Smad pathways. First studies suggested that activation of Wnt/Notch pathways results in



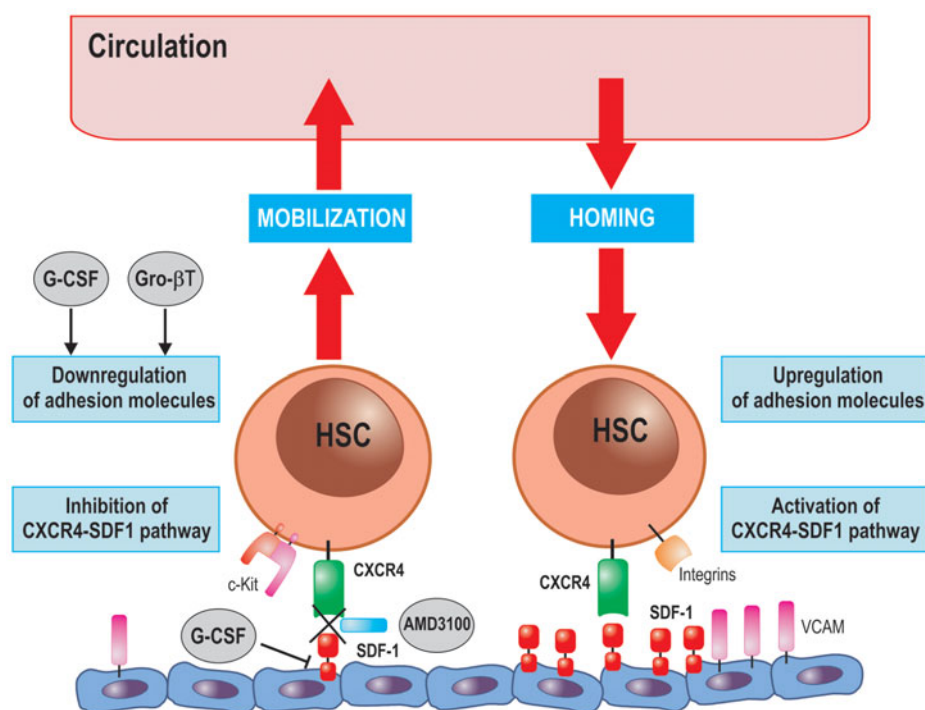
increased proliferation and self-renewal of HSCs,<sup>26-28</sup> and expression of the Wnt inhibitor Dickkopf-1 results in reduced *in vivo* repopulating ability, and loss of quiescence. But recent data in various genetic models make it unlikely that either of these two pathways play a major role in HSC maintenance.<sup>29,30</sup> In a similar way, HSC cultured *in vitro* with Sonic hedgehog (Shh) promotes proliferation but studies in mice<sup>31</sup> showed that prolonged signaling needs to be controlled in order to prevent HSC exhaustion and loss. Moreover, treatment with the inhibitor cyclopamine is well tolerated, showing that this signaling is not absolutely critical for HSC maintenance. The Smad signaling pathway functions to transduce signals downstream of the transforming growth factor- $\beta$  (TGF- $\beta$ ) family of ligands, such as TGF- $\beta$ , activins and bone morphogenetic proteins (BMPs). Recently, several reports have shed new light suggesting that this pathway plays a pivotal role in the regulation of HSC fate decisions. TGF- $\beta$  is one of the most potent inhibitors of the HSC growth *in vitro*, but exits an apparent discrepancy between *in vitro* and *in vivo* findings, so mice deficient of the TGF- $\beta$ -Type I receptor display normal HSC self-renewal and regeneration capacity.<sup>32</sup> In the same way, BMP-4 was shown to promote maintenance of adult HSC in culture but does not appear to affect proliferation of purified murine HSC.<sup>33</sup> Thus, it is proposed that other ligands such as activin take part in the signaling through the same Smad pathway.

While it is widely assumed that HSC require cell-cell contact within the niches, the nature of the environment *per se* could be essential for the maintenance and protection of HSCs. There are some evidences that exposure to high levels of oxygen negatively affects HSCs, such as diminished self-renewal and an increase in cell-cycle entry. Hypoxia is regarded as an essential component for proper stem cell function *in vivo*, although the underlying molecular mechanisms remain to be elucidated. Some studies showed that hypoxic conditions enhance “stemness” by HIF-1 $\alpha$  mediated regulation of genes involved in cell-cycle control. In addition, HSCs lacking the calcium-sensing receptor (CaR) fail to engraft in the bone marrow suggesting that high calcium concentrations are necessary for the activity of the endosteal niche.<sup>34</sup> The bone marrow niche is a dynamic system, in part mediated by circadian oscillations in sympathetic nervous system tone. Adrenergic inputs effect stem cell mobilization through down-regulation of CXCL12 during daytime<sup>35</sup> and up-regulation of CXCR4 expression on HSC at night,<sup>36</sup> with consequent changes in mobilization of HSC into the peripheral blood. Clinically, the timing of HSCs harvest or infusion may impact the yield or engraftment, respectively, and may result in better therapeutic outcomes.

In summary, the molecular events that occurs in HSC regulation emphasize the complexity of physiological conditions under which the niche orchestra all the signals into a delicate balance between self-renewal and differentiation.

## MECHANISMS OF MOBILIZATION AND HOMING

The relationships between hematopoietic cells and their niches are highly dynamic and thus, may support rapid increase in hematopoietic cell production depending on the physiological requirements. Migration of HSCs is based in the mechanisms that enable to these cells to leave the bone marrow and enter the circulation (mobilization), relocate to a distant tissue and return to the bone marrow (homing). HSC exit the bone marrow by migration through the bone marrow-blood barrier, and then become disseminated in the circulation. Once blood-borne, HSCs may relocate into a target tissue or home back



**Figure 3.** Hematopoietic stem cells mobilization and homing in the bone marrow. Mobilization and Homing are mirror images and opposite processes involving the same molecules but in opposite directions. Down-regulation of adhesion molecules and chemokine receptors on HSCs drive the mobilization of these cells to the blood. In the other way, HSCs which express the chemokine CXCR4 are chemoattracted to the highly expressing SDF-1 endosteal niche. Several cytokines and new agents are showed to induce the mobilization of HSC from bone marrow to peripheral blood stream. G-CSF and Gro $\beta$  induces the release of proteolytic enzymes-like neutrophil elastase, cathepsin G and MMP-9, which finally downregulate adhesion molecules and inactivate SDF-1 by cleavage N-terminal signal sequence. Moreover, G-CSF decreases SDF-1 mRNA expression in a protease-independent mechanism. The new agent AMD3100, reversibly and selectively blocks SDF-1 binding to CXCR4 resulting in rapid mobilization of HSCs.

to the bone marrow compartment with high efficient. BM homing is a prerequisite for consecutive HSC proliferation and it is of pivotal importance during fetal development and for HSC homeostasis in adulthood.

HSC mobilization and homing are mirror processes regulated by the interplay of cytokines, chemokines and proteases (Fig. 3). HSC mobilization is characterized by loss of cell-cell contacts (downregulation of cell adhesion molecules) and a desensitization of chemokine signaling, mainly the SDF-1/CXCR4 axis. Conversely, stem cell homing requires upregulation of cell adhesion molecules and activation o SDF-1/CXCR4 signaling pathway.

### Mobilization from Bone Marrow

During embryogenesis, blood-forming stem cells migrate from the fetal liver via the circulation, home to the bone marrow, and repopulate it with high numbers of immature

and maturing blood cells of all lineages. These, in turn, are released into the circulation and reached other body sites (spleen and liver) while maintaining a small pool of undifferentiated stem cells within the bone marrow, suggesting that there is a dynamic movement of stem cells from the bone marrow to other body sites. Mobilization of HSCs from bone marrow is tightly regulated by specialized signals, involving cytokines, chemokines, growth factors and hormones.

Principally, SDF-1/CXCR4 signal plays a pivotal role for rapid mobilization of HSC from bone marrow niches. Under homeostatic conditions, most HSCs remain in the bone marrow niches retained by high SDF-1 expression, which is maintained by the hypoxic microenvironment. In this way, local hypoxia leads to upregulation of SDF-1 expression on endothelial cells,<sup>37</sup> which mediate recruitment of HSCs to sites of vascular injury. However, after an ischemic insult, SDF-1 is released from injured tissue and stimulates mobilization of HSCs from the bone marrow to ischemic tissue. Thus, SDF-1 is the main chemokine for initiating stem cell migration and the majority of cytokines that mediate stem cell migration do so via modulation either of SDF-1 or of this receptor, CXCR4.

The proteases, Elastase and Cathepsin G are also involved in the release of HSCs to the circulation in human.<sup>38,39</sup> Elastase plays a major role in dislodging HSCs from BM stroma by degrading the adhesion molecules VLA-4 and VCAM. Furthermore, these proteases also degrade CXCR4 and SDF-1 at the amino-terminal residue of both molecules. Other proteases, such as the matrix metalloprotease (MMP)-9 had also been implicated in the mobilization process. Activation of MMP-9 results in degradation of anchorage proteins, enabling migration of HSCs from osteoblastic to the vascular niche. MMP-9 cleaves the HSC mobilizing factor kit-ligand from the membrane-associated moiety and degrades osteopontin, inducing HSC cycling and reducing anchorage to the osteoblastic niche.<sup>40</sup> Moreover, circulating VEGF induced MMP-9 expression in the BM leading to the release of soluble Kit ligand. In addition, IL-8 has been proposed to use matrix metalloprotease (MMP)-9 activity, and induce mobilization, although how exactly this impacts on SDF-1/CXCR4 axis is not fully understood. However, studies in mice lacking of functional serine proteases showed that a protease-independent pathway also contribute to HSC mobilization. Other signaling molecules, such as nitric oxide (NO) and oxygen had been recently implicated in the mobilization of HSC. Mice deficient in endothelial NO synthase (eNOS) showed reduced mobilization of CD34<sup>+</sup> cells compared with wildtype mice.<sup>41</sup>

As it will be further described in detail, the cytokines G-CSF/GM-CSF and chemotherapy induce the HSCs mobilization by disruption of cell-cell contacts by cleavage of cell adhesion molecules and simultaneously has a negative effect on CXCR4-SDF1 signaling by dual inactivation of both the receptor and the ligand.

### **Homing to the Bone Marrow or Target Tissue**

A prerequisite for proper HSC function is the ability to travel through the blood stream and find these specialized bone marrow niches, in a process called homing. Homing constitutes a multistep cascade comprising: Recognition and interaction with microvascular endothelium, transmigration through the vascular endothelium, and finally, migration and invasion of the target tissue. This process, similar to mobilization, relies on a complex interplay between cytokines, chemokines, adhesion molecules, and extracellular matrix-degrading proteases.

HSCs are required to migrate across the vascular endothelium, a process known as extravasation. The first response to endothelial disruption is platelet adhesion, which has been shown to target HSCs to sites of endothelial disruption. Circulating HSC show low affinity contacts with the endothelial cells monolayer in order to get activated by cytokines and to arrest in the next step on the endothelial cells. This process called rolling is predominantly mediated by E- and P-selectins and its ligands. E-selectin deficient mice showed impaired homing and recovery after ischemia, but surprisingly this was reversed by the soluble molecule (sE-selectin) indicating that the shed of E-selectin is regulating the recruitment of HSCs.<sup>42</sup> Soluble E-selectin also stimulates the expression of the adhesion molecules ICAM-1 and VCAM-1 on the endothelial cells to increase the homing. Additionally, HSC are adhered directly to P-selectin on platelets and endothelial cells improving the vascularization.<sup>43</sup> Subsequent firm attachment on the endothelium is mediated by integrins, such as the ICAM-1/LFA-1, VCAM/VLA-4 ligand pairs. VLA-4 was first described as a lymphocyte antigen and adheres to VCAM and fibronectin on new endothelium. These interactions have been reported to be critically important because HSC homing was inhibited at least 90% by blocking antibodies to VLA-4 ( $\alpha 4\beta 1$  integrin) or VCAM-1.<sup>44</sup> SDF-1 increases the adhesion of the integrins VLA-4 and LFA-1 to their respectively endothelial ligands.<sup>45</sup>

Transendothelial migration and invasion of cells requires degradation of basement membrane, which is dependent on the production of matrix-degrading enzymes; especially MMPs. These proteases may act by degrading the extracellular matrix that is necessary for tissue invasion, but additionally may regulate cytokines, receptors and ligands in ischemic tissue, limiting the engraftment. MMP-2 was shown to cleavage and inactivates SDF-1 and its receptor CXCR4<sup>46</sup> which are essential for the recruitment. Moreover, VEGF is a target of MMP-3 and MMP-9<sup>47</sup> indicating that MMPs may play a double-edged role. These data had carried to speculate that MMPs might be specifically involved in HSCs mobilization and homing to tissue target.

Additionally, other interactions are also essential for retaining HSCs in the bone marrow. Indeed, overexpression of SDF-1 plays a crucial role for recruitment of circulating or intravenously infused cells.<sup>48</sup> Studies in animals showed that mice deficient in either SDF-1 or CXCR4 die during late embryogenesis,<sup>49</sup> and CXCR4-blocking antibodies impair HSC engraftment.<sup>50</sup> Surprisingly, two independent groups show that HSCs isolated from the fetal liver of mice with homozygous deletion of the CXCR4 genes (CXCR4<sup>-/-</sup>) homed normally in the bone marrow when injected intravenously into lethally irradiated adult mice, suggesting that CXCR4 is dispensable for HSCs homing to adult bone marrow.<sup>51,52</sup> However, eight weeks after, the number of circulating HSCs in the blood was dramatically increased in these mice, showing that CXCR4 is necessary for efficient HSC retention within the bone marrow.

In addition to this, there is a local synthesis and expression of SDF-1 and VEGF, which are directly regulated by tissue oxygen tension. SDF-1 is constitutively expressed in the bone marrow endothelia, but in nonhaematopoietic sites requires hypoxia-inducible factor-1 (HIF-1) activation. Therefore, hypoxic microenvironments might constitute transient ischemic niches at sites of tissue damage, directing organ-specific homing of circulating HSCs.<sup>37</sup> The VEGF family is the primary mediator of the angiogenic switch, enabling revascularization of ischaemic tissue. Local VEGF levels promote organ-specific HSCs homing and positioning close to newly forming vessels.<sup>53</sup>

In short, the SDF-1/CXCR4 chemotactic axis and VCAM-1/VLA-4 adhesive interaction appear to be two important no redundant pathways retaining HSCs within the bone marrow and obvious targets for therapies in order to enhance HSC mobilization.

## AGENTS FOR HSC MOBILIZATION

Multiple randomized trials have demonstrated the benefits of peripheral blood stem cells (PBSC) over BM, including less invasive collection methods, reduced morbidity, faster engraftment, shorter hospitalization and lower total cost. In this way, and due to HSCs circulate in the peripheral blood in very low numbers (0.01-0.05%), mobilization is necessary to drive sufficient numbers of HSCs from the BM to the peripheral circulation, where they can be harvested by apheresis.

Currently, the two most common mobilization strategies use cytokines alone or cytokines after chemotherapy (chemomobilization). The hematopoietic growth factors GM-CSF and G-CSF developed 20 years ago and approved by the FDA (US Food and Drug Administration) are the most commonly used mobilization agents.<sup>54,55</sup> Mobilization with these cytokines is generally well tolerated but some patients and donors fail to mobilize adequate number of HSCs and alternative strategies are required. In the last ten years the major understand of the interactions between stem cells and BM microenvironment had allowed that new agents for HSCs mobilization are being developed.

### Cytokines/Chemotherapy

#### *G-CSF*

Currently, recombinant human G-CSF produced in *Escherichia coli* is the agent most commonly used to elicit HSC mobilization in the clinic. Administration of G-CSF increase the number of myeloid cells, which release active neutrophil serine proteases such as cathepsin G and neutrophil elastase, along with matrix metalloproteinase-9 into the extracellular fluid on BM.<sup>40,56</sup> This results in enhances cleavage of the adhesion molecules VCAM-1, c-kit, CXCR4 and SDF-1. A recent study suggests that G-CSF could regulate the mobilization of HSCs in a protease-independent mechanism, because G-CSF reduces osteoblasts activity and decrease SDF-1 mRNA expression<sup>57</sup> (Fig. 3). Although the biological mechanisms are not fully understood and probably there is a more complex model in which both protease-dependent and protease-independent pathways take place.

An interested effect of G-CSF-mobilized blood cells observed in murine models with full HLA-mismatched transplants is the skewing of donor T cells to a Th2 profile with higher secretion of IL-10, and lower secretion of IL-4 and interferon- $\gamma$ .<sup>58</sup> Consequently, infusion of G-CSF-mobilized donor CD4<sup>+</sup> T cell attenuates graft-versus-host disease (GVHD).

G-CSF is injected daily at doses ranging from 10 to 32  $\mu\text{g/kg}$  s.c beginning at least 4 days before the first apheresis session and continued until the last session. It is known that CD34<sup>+</sup> cell levels peak on the 5th day after administration of G-CSF. It is assumed that the infusion of  $2 \times 10^6$  CD34<sup>+</sup>/kg results in adequate engraftment, but a dose of  $>5 \times 10^6$  CD34<sup>+</sup>/kg produces more rapid and predictable hematopoietic and specifically platelet recovery. Generally, G-CSF is well tolerated by patients, with few side effects apart from bone pain experienced by around 80% of donors; however, 5-10% of normal allogeneic donors will fail to mobilize sufficient HSCs for optimal transplantation and this can increase up to 40% in the autologous transplants. The variability in the response is multifactorial and may involve poor granulocyte activation, lower protease content, or reduced HSC pool size. Several salvage regimens have been

developed to improve mobilization in these patients, including high-dose G-CSF and combinations with other cytokines, such as GM-CSF,<sup>59</sup> although this increase the risks associated with treatments.

Other of the problems with recombinant G-CSF, produced in bacteria and therefore unglycosylated, is the relatively rapid plasma clearance (4-6 h half-life) being necessary daily injections. To overcome these difficulties, a recombinant G-CSF has been chemically conjugated to polyethylene glycol (pegylated), which slows plasma clearance. Pegylated G-CSF (Pegfilgrastin) has a half-time of 33 h due to a slow rate of renal elimination, and has the capacity to decrease the doses of product to induce mobilization and the length of time to collect enough HSCs.<sup>60</sup> Administration of Pegfilgrastim has been approved by the FDA for prevention of prolonged neutropenia after chemotherapy for nonhematological malignances and is well tolerated, with adverse effects similar to that of G-CSF.

### *GM-CSF*

Induces mobilization by stimulating proliferation and differentiation of hematopoietic progenitor cells, particularly CD14<sup>+</sup> monocytes and CD80<sup>+</sup> dendritic cells. Although it was approved for mobilization, nowadays GM-CSF is little used for that purpose due to a higher incidence of both mild and serious adverse events.

### *Chemotherapy*

Before the development of hematopoietic cytokines as mobilization agents, the earliest protocols to mobilize HSCs into the peripheral blood used chemotherapy alone.<sup>61</sup> When G-CSF and GM-CSF became available, daily administration of either agent after chemotherapy was found to enhance HSC mobilization. Chemomobilization results in higher collection of HSCs, which may promise faster engraftment of platelets and neutrophils and improved rates of survival. Some researchers have speculated that mobilization with chemotherapy plus G-CSF may have a synergistic effect on protease release in the BM, which cleave the adhesion molecules SDF-1, CXCR4 and c-kit. In addition, the toxicity of chemotherapeutic agents to the BM stroma could facilitate the release of HSCs by damaging the functional ability of stromal cells to support stem cells.

A variety of chemotherapeutic agents are used with cytokines to mobilize HSCs for autologous transplantation. Administration of CY with G-CSF is widespread, and requires fewer apheresis sessions to collect sufficient number of HSCs than mobilization with G-CSF alone. The benefits of adding chemotherapeutic agents to a G-CSF mobilization regimen may be offset by the increased risk of complications to the patients,<sup>62</sup> associated with increased morbidity, greater risk of infection, more hospital admissions, transfusions, antibiotic therapy and considerably greater cost overall.

### **Novel Agents**

Some patients and donors fail to mobilize adequate number of HSCs with the standard approaches described above, and new mobilization strategies need to be developed. Several new cytokines and chemokines have emerged as potential agents for stem cell mobilization, including stem cell factor (CSF) and Gro $\beta$ , among others.



Most mobilizing agents target accessory cells (granulocytes, osteoblasts, stromal cells) rather than the HSCs themselves. The chemokine receptor CXCR4 is an ideal target to directly mobilize HSCs, since it is expressed by all HSCs and its function is absolutely required for their retention within the bone marrow. Two types of CXCR4 antagonists have been developed: Exopeptidase-resistant CXCL12-mimetic peptides (CTCE-0021 and CTCE0214) and small synthetic chemicals, in particular AMD3100.

#### *CTCE0021 and CTCE0214*

Are small cyclized peptides analogues (31 amino acids) hybrid of the N-terminal and C-terminal regions of SDF-1 linked to four-glycines linker, which were shown to have biological function comparable to the native molecule.<sup>63,64</sup> Both analogues induced enhanced chemotaxis of normal and G-CSF-mobilized HSCs, in a dose-dependent manner. Following an injection of CTCE0214 into mice, an increase in HSCs was detected as early as 4 h after injection, and became significant at 24 h. One study suggest that cord blood CD34<sup>+</sup> cells cultured in vitro with CTCE0214 showed an increased survival and when these cultured cells were transplanted into NOD/SCID mice a more engraftment of human cells into bone marrow was reached.<sup>65</sup> In this way, SDF-1 peptide analogue CTCR0214 could be explored for the potential use of ex-vivo expansion of cord blood CD34<sup>+</sup> cells for transplantation.

#### *AMD3100 (Plerixafor)*

Is a reversible inhibitor of CXCR4-SDF-1 binding which mobilizes CD34<sup>+</sup> cells into the peripheral blood<sup>66</sup> (Fig. 3). It was initially developed as a potential treatment for HIV, blocking the HIV entry into CD4<sup>+</sup> T cells by binding to the HIV coreceptor CXCR4.<sup>67</sup>

In normal volunteers, the administration of AMD3100 after 4-5 days of G-CSF resulted in a 3 to 3.5 fold increase in circulating CD34<sup>+</sup> cells, and fewer aphaeresis were needed.<sup>68,69</sup> A single dose of AMD3100 at 160-240 µg/kg sc resulted in 6-10 fold increase in CD34<sup>+</sup> cell count starting 1 hour, peaking at 9 hours after injection and declining to baseline within 24 hours.

HSCs mobilized with AMD3100 plus G-CSF showed increased expression of CD49d and decreased expression of CD62L (L-selectin) which suggest the acquisition of an “engrafting” phenotype.<sup>70</sup> Furthermore, expression profiling of HSCs mobilized with G-CSF plus AMD3100 showed increased expression of genes protective against apoptosis, and regulating cycle, DNA repair and oxygen transport, compared with CD34<sup>+</sup> cells mobilized by G-CSF alone.<sup>71</sup> Interestingly, as CXCR4 is expressed by many tumor cells, there is the potential risk that malignant cells are also mobilized in the autologous setting. This effect, however, could be counter-balanced by an increase in chemosensitivity of malignant cells mobilized with AMD3100.<sup>72</sup> Thus, AMD3100 could also be a useful adjunct to existing chemotherapy treatments.

In two Phase III studies in patients with multiple myeloma (MM) and non-Hodgkin's lymphoma NHL<sup>73,74</sup> confirmed that the addition of AMD3100 to G-CSF is generally safe and well tolerated. Adverse effect more frequently were transient pain and injection site erythema, headache, paresthesias, diarrhea, bloating and nausea. Plerixafor (Mobozil®, Genzyme Corp.) had been approved by the FDA and the European Medicines Evaluation Agency (EMA) to enhance stem cell mobilization for autologous transplant in patients with lymphoma and multiple myeloma.<sup>75,76</sup>



### *Stem Cell Factor (SCF)*

Is a cytokine that acts on primitive multilineage hematopoietic cells and stimulated mobilization of myeloid, erythroid and lymphoid progenitors. In the majority of trials SCF acts synergistically with G-CSF, significantly reducing number of leukaphereses required to collect CD34<sup>+</sup> cells collected.<sup>77</sup> But, severe anaphylactoid reaction occurs in 5-10% of patients receiving SCF and thus it has not been FDA-approved in USA, although it is approved for use in Canada and New Zealand.

### *GRO-β (CXCL2)/SB-251353*

Gro-β is a chemokine which exerts its biological activity by binding to the CXCR2 receptor. SB-251353 is a recombinant, N-terminal truncated form of human Gro-β which binds to CXCR2 receptor with greater potency than the full length form of Gro-β.<sup>78</sup> Studies in murine and nonprimate models showed rapid mobilization of stem cells and acts synergistically with G-CSF,<sup>79</sup> but further studies are necessary to determine the efficacy and potential toxicities of this treatment in humans.

### *GROβT (CXCL2Δ4)*

GroβT mobilizes HSCs into the peripheral blood in an equivalent fashion to G-CSF when used alone, and synergizes with G-CSF when used in combination.<sup>80</sup> Enhanced engraftment was not due to increased numbers of transplanted short-term repopulating cells, their homing/migratory potential, or adhesion molecules expression; however may result from the selective mobilization of earlier long-term repopulation cells, or cells with an intrinsic capacity for accelerated engraftment and proliferation. The mobilization effect of GroβT is hypothesized to be mediated by up-regulation of plasma pro-MMP-9, altering pro-MMP-9: TIMP-1 (tissue inhibitor of metalloproteinase-1) stoichiometry favoring MMP-9 activation. When GroβT is used in combination with G-CSF an increase of active plasma MMP-9 was observed, and mobilization is fully blocked by anti-MMP-9 and is absent in MMP-9 deficient mice.

### *Parathyroid Hormone (PTH)*

Poor mobilization response can also due to a damaged microenvironment or depleted HSC number. In this way, strategies that increase total HSC number in the bone marrow should also lead to enhanced HSC mobilization. Some studies have confirmed that HSC number is increased in mice carrying transgenes that enhance bone formation. Furthermore, osteoblast function is inhibited during G-CSF-induced mobilization, possibly associated with the bone pain experienced by most donors. These findings suggest that treatments stimulating bone formation may increase the number of HSCs in the bone marrow, which in turn should result in enhanced mobilization.

Recently, studies using daily injections of parathyroid hormone (PTH) for 5 weeks, directly increases bone formation, found a doubling in the numbers of HSCs mobilized into the blood in response to G-CSF as well as higher levels of engraftment.<sup>81</sup> In a Phase I study, 20 patients who previously had a poor mobilization response to G-CSF, received escalating doses of PTH (40-100 µg s.c) for 14 days and combined with G-CSF 10 µg/kg on the last 4 days of treatment.<sup>82</sup> Overall, 47% of patients with one previous failure and

40% of patients with two failures reached the mobilization criterion of  $>5 \text{ CD34}^+$  cells/ $\mu\text{l}$  in the peripheral blood. PTH was well tolerated and adverse effect included headache, muscle pain, back pain, fatigue and hypothermia.

## CONCLUSION AND FUTURE PROSPECTS

Hematopoietic stem cells are located in the bone marrow in specialized areas called niches. During homeostasis several signals from the microenvironment keep some HSCs in a quiescent state, whilst other HSCs are activated in order to maintain haematopoiesis. Although significant progress had been done in the last years to understand the cellular and molecular components of the niches, several important questions remain unresolved.

The success of hematopoietic stem cell transplantation is influenced by a number of factors, being the dose of re-infused stem cells a key factor. For this HSC need to be mobilized from the bone marrow to peripheral blood, where they are easily collected and after administer via intravenous injection. Mobilization agents currently used in clinic, mainly G-CSF cytokine, are well tolerated but often produce suboptimal number of collected HSCs. A better understanding of the mechanisms and identification of the factors that directly regulates the migration process will be necessary to enhance present strategies involving stem cells engraftment.

Recent advances have shown that HSC mobilization can be enhanced by at least two different approaches. First, target the chemotactic interaction between HSCs and supportive stromal cells with CXCR4 antagonists. AMD1300 (Plerixafor) is a direct inhibitor of CXCR4 and mobilized HSC within hours. Several clinical trials have shown strong synergism between CXCR4 antagonists and G-CSF for stem cell mobilization. But when the main problem is reduced HSC numbers in the bone marrow, the strategy is to enhance HSC content by enhancing bone formation prior to mobilization. Some studies with TPH had been carried with good results but the future will now be to find small synthetic molecules that can stimulate HSC expansion more rapidly and at a lower cost than PTH.

The future of mobilization will use promising new agents in the context of a patient-tailored strategy that depends on individual disease characteristics and the nature of previous treatment.

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# GREAT PROMISE OF TISSUE-RESIDENT ADULT STEM/PROGENITOR CELLS IN TRANSPLANTATION AND CANCER THERAPIES

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**Abstract:** Recent progress in tissue-resident adult stem/progenitor cell research has inspired great interest because these immature cells from your own body can act as potential, easily accessible cell sources for cell transplantation in regenerative medicine and cancer therapies. The use of adult stem/progenitor cells endowed with a high self-renewal ability and multilineage differentiation potential, which are able to regenerate all the mature cells in the tissues from their origin, offers great promise in replacing non-functioning or lost cells and regenerating diseased and damaged tissues. The presence of a small subpopulation of adult stem/progenitor cells in most tissues and organs provides the possibility of stimulating their in vivo differentiation, or of using their ex vivo expanded progenies for cell-replacement and gene therapies with multiple applications in humans without a high-risk of graft rejection and major side effects. Among the diseases that could be treated by adult stem cell-based therapies are hematopoietic and immune disorders, multiple degenerative disorders such as Parkinson's and Alzheimer's diseases, Types 1 and 2 diabetes mellitus as well as skin, eye, liver, lung, tooth and cardiovascular disorders. In addition, a combination of the current cancer treatments with an adjuvant treatment consisting of an autologous or allogeneic adult stem/progenitor cell transplantation also represents a promising strategy for treating and even curing diverse aggressive, metastatic, recurrent and lethal cancers. In this chapter, we reviewed the most recent advancements on the characterization of phenotypic and functional properties of adult stem/progenitor cell types found in bone marrow, heart, brain and other tissues and discussed their therapeutic implications in the stem cell-based transplantation therapy.

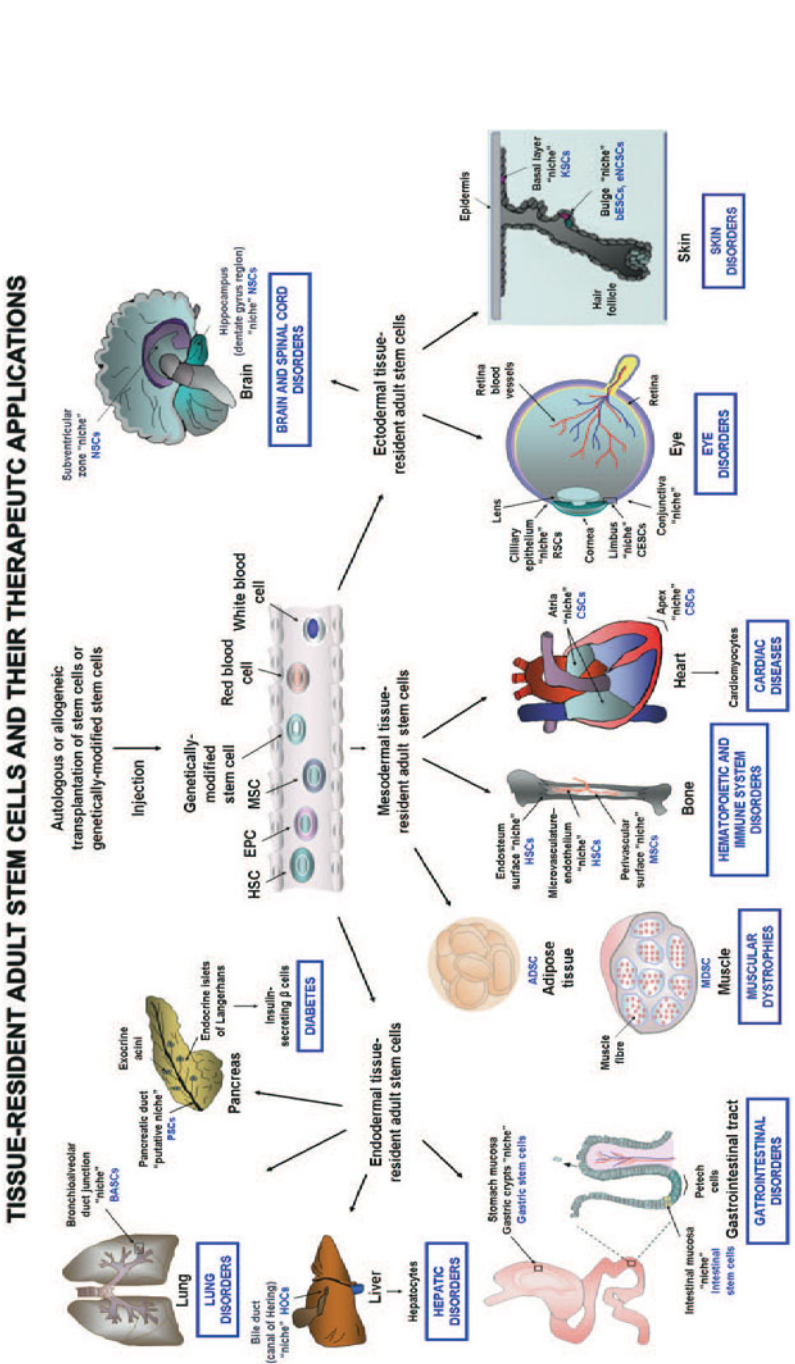


## INTRODUCTION

Recent advances in the field of the stem cell biology have led to the characterization of different tissue-resident adult stem/progenitor cells in most mammalian tissues and organs that constitute potential and easily accessible sources of immature cells with multiple promising therapeutic applications in stem cell-based transplantation therapies. Among the tissues harboring a small subpopulation of adult stem/progenitor cells, there are bone marrow (BM), vascular walls, heart, brain, tooth, skeletal muscles, adipose tissues as well as the epithelium of the skin, eye, lung, liver, digestive tract, pancreas, breast, ovary, uterus, prostate and testis (Fig. 1).<sup>1-14</sup> Numerous studies have allowed researchers to define the unique features of each tissue-resident adult stem/progenitor cell type and their specialized local microenvironment designated as a niche (Fig. 1).<sup>1-4,6-15</sup> The tissue-resident adult stem/progenitor cells and their early progenies endowed with a high self-renewal and multilineage differentiation potential generally provide critical physiological functions in the regenerative process for tissue homeostatic maintenance, and repair after intense injuries, such as chronic inflammatory atrophies and fibrosis.<sup>1-4,6-14</sup> Multipotent adult stem/progenitor cells are able to give rise to different differentiated cell lineages in tissues from which they originate in physiological conditions, and thereby regenerate the tissues and organs throughout the lifespan of an individual. Importantly, it has also been shown that certain adult stem/progenitor cells, including BM-derived stem/progenitor cells, may be attracted at distant extramedullary peripheral sites after intense injuries, and thereby participate in the tissue repair through remodeling and regeneration of damaged areas.<sup>1,2,9-11,14-17</sup>

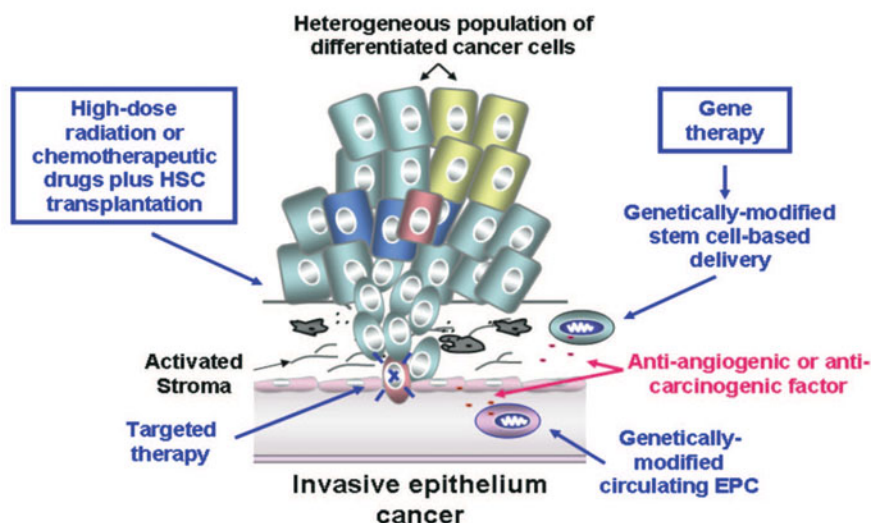
Of clinical interest, it has been shown that the small pools of endogenous adult stem/progenitor cells can be successfully used for cell replacement-based therapies in regenerative medicine and cancer therapy in humans.<sup>3,9-11,14,16-36</sup> The use of autologous adult stem/progenitor cell transplant may reduce the high-risk of graft rejection and severe secondary effects observed with allogenic transplant or embryonic stem cell (ESC)-based transplantation therapies. Particularly, the *in vivo* stimulation of endogenous tissue-resident adult stem/progenitor cells or the replacement of nonfunctioning or lost adult stem/progenitor cells by new *ex vivo* expanded immature cells or their differentiated progenies have been recognized as promising therapeutic strategies.<sup>3,9-11,14,16-21,23-36</sup> Among the human diseases that could be treated by stem cell-based transplantation therapies, there are hematopoietic and immune disorders, Type 1 or 2 diabetes mellitus, cardiovascular, neurodegenerative and musculoskeletal diseases and skin, eye, tooth, liver, lung, and gastrointestinal disorders and aggressive and recurrent cancers (Figs. 1 and 2).<sup>3,9-11,14,16-21,23-36</sup> In regard with this, we discussed the most recent progress in basic and clinical research in the adult stem/progenitor cell field in terms of their implications in the development of novel stem cell-based transplantation therapies. The emphasis is on the phenotypic and functional properties of adult stem/progenitor cells found in BM, heart and brain and their potential therapeutic applications to treat diverse severe disorders and aggressive cancers.





**Figure 1.** Scheme showing the anatomic localizations of tissue-resident adult stem/progenitor cells and their niches and adult stem cell-based transplantation therapies for treating diverse human disorders. The clinical treatments consisting of an injection of autologous or allogeneic adult stem/progenitor cell transplant, including bone marrow (BM)-derived stem cells (HSC, EPC, MSC), peripheral blood (PB) or genetically-modified adult stem/progenitor cells into peripheral circulation or diseased areas in the same patient or a host patient is illustrated. The tissue-specific degenerating disorders and diseases which might be treated by the autologous or allogeneic transplantation of adult stem/progenitor cells are also indicated.

## ADULT STEM/PROGENITOR CELL TRANSPLANTATION THERAPIES AGAINST AGGRESSIVE AND RECURRENT CANCERS



**Figure 2.** Scheme showing potential combination therapies against locally invasive and metastatic epithelial cancers. The therapeutic strategies consisting of targeted therapy of tumor-initiating cells and their local microenvironment, including stromal components, high-dose radiation or chemotherapy plus adult stem/progenitor cell transplantation and selective delivery of anti-tumoral drugs by using genetically-modified stem cells, are also illustrated.

## BONE MARROW-DERIVED STEM/PROGENITOR CELLS AND THEIR THERAPEUTIC APPLICATIONS IN TRANSPLANTATION THERAPIES

### Hematopoietic Stem/Progenitor Cells and Their Clinical Applications

The BM-resident hematopoietic stem cells (HSCs) provide critical functions for the maintenance of hematopoiesis and the immune system by generating all of the mature myeloid and lymphoid cell lineages in the peripheral circulation along the lifespan of an individual.<sup>1,9-11</sup> The most immature and quiescent HSCs are colocalized with the osteoblasts in a specialized niche within a BM region designated as endosteum (Fig. 1).<sup>1,2,9,11,12</sup> These multipotent HSCs are characterized by the expression of specific biomarkers including telomerase, high levels of aldehyde dehydrogenase and CD34<sup>-</sup> or CD34<sup>+</sup>/CD38<sup>-low</sup>/Thy1<sup>+</sup>/CD90<sup>+</sup>/C-kit<sup>-lo</sup>/Lin<sup>-</sup>/CD133<sup>+</sup>/vascular endothelial growth factor receptor 2 (VEGFR2<sup>+</sup>)/ABCG2 multidrug transporter. Moreover, another HSC subpopulation found in a BM microvasculature-sinusoidal endothelium niche also can contribute to rapidly supplying new mature blood cell lineages, which have a short life in the peripheral circulation (Fig. 1).<sup>1,11</sup> In regard with this, the results from a recent study have also revealed the presence of postnatal CD34<sup>+</sup>/Lin<sup>-</sup>/CD10<sup>+</sup>/CD24<sup>-</sup> hematopoietic progenitor cells co-expressing recombination activating gene 1, terminal deoxynucleotide transferase, paired box protein 5 (PAX5), interleukin 7 receptor- $\alpha$  and CD3 $\epsilon$  in the BM and peripheral blood.<sup>37</sup> These hematopoietic progenitor cells, which

exhibited a very low potential along myeloid commitment, were able to migrate from BM to thymus and generate B-, T-, and natural killer (NK)-lymphocytes.<sup>37</sup> Additionally, the primitive KIT<sup>+</sup> hematopoietic progenitor cells endowed with a long-term self-renewal capacity have also been identified in the adult spleen in humans.<sup>38</sup> The activation of HSCs and their early progenitors implicates the interplay of a complex signaling network mediated by different growth factors including hedgehog, Wnt/ $\beta$ -catenin, Notch, fibroblast growth factor (FGF) and Polycom group proteins, such as BMI-1 and interactions with the niche components that control their self-renewal ability versus differentiation capacity.<sup>1,2,9,11,12,39</sup>

In clinical practice, autologous or allogeneic HSC transplantation is currently used to treat the patients with diverse hematopoietic disorders to reconstitute the hematopoietic cell lineages and immune system defense.<sup>9-11,18,19</sup> BM-derived HSCs may be collected from BM aspirate or by aphaeresis after their mobilization in peripheral blood (PB) by using diverse mobilizing agents such as granulocyte-stimulating factor (G-CSF), granulocyte colony-stimulating factor (GM-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF) and/or synthetic chemical compounds-like bicyclam derivative, AMD3100 (Plerixafor).<sup>9,11,40</sup> Hence, BM or mobilized PB HSC-containing samples or isolated HSC preparations may be retransplanted into the same patients (autografts) or different patients (allografts) by injection into the bloodstream (Fig. 1). Transplanted HSCs spontaneously migrate and engraft at the BM compartment where they establish their novel homing, and thereby contribute to replenish all the mature blood cell types and restore immune system functions. Moreover, a treatment of patients with a myeloablative conditioning regimen consisting of high-dose chemotherapy or radiotherapy is generally made prior to allogeneic HSC transplantation to reduce the immune response and risk of graft rejection and improve the anti-tumoral efficacy. The HSC transplants, alone or in combination therapies, may be used to treat HSC aging related-intrinsic functional defects, inherited immunodeficient and autoimmune diseases such as multiple sclerosis, refractory and severe aplastic anemias, congenital thrombocytopenia, osteoporosis, cardiovascular disorders, chronic inflammatory Bowel disorders (IBDs) including Crohn's disease and ulcerative colitis, and diabetes mellitus.<sup>9-11,20,21</sup> Particularly, HSC transplant may improve the immune response of patients, and thereby help to repair damaged tissues at distant sites in diverse pathological conditions and prevent infectious diseases after the transplantation of tissue or organ grafts.<sup>9,11,18</sup> Moreover, high-dose or intermittent systemic chemotherapy or ionizing radiation therapy plus HSC transplantation represents a potential therapeutic option to treat and even cure the high risk patients with advanced and/or relapsed cancers (Fig. 2). Among them, there are leukemias, multiple myeloma, Hodgkin's and non-Hodgkin's lymphomas, melanoma and aggressive and metastatic solid tumors such as sarcomas, retinoblastoma, kidney, brain, lung, pancreatic, prostate, breast and ovarian cancers.<sup>9-11,19,41,42</sup> In fact, HSC transplant may restore the hematopoietic and immune systems after myeloablative effects induced by high-dose irradiation or chemotherapy following a treatment of cancer patients (Fig. 2).<sup>19</sup>

Although there have been important advances in HSC transplantation procedures, the graft-versus-host diseases (GVHDs), toxicity of cytoreductive conditioning regimens, the presence of residual malignant cells in allograft as well as the lack of appropriate donors for some patients, represent the major limiting factors for their clinical applications in safe conditions.<sup>43</sup> Particularly, several secondary effects may be manifested after HSC transplantation in certain patients and contribute to a poor quality of life.<sup>44</sup> For example,

acute or GVHD is a common late complication of allogeneic transplantation characterized by specific clinical and pathologic signs related with the fact that immunocompetent donor cells may attack to fast proliferating recipient tissues such as the skin, liver and gastrointestinal tract.<sup>45</sup> GVHD may be associated with the occurrence of severe vascular and fibrotic lesions. In order to reduce the toxicity of myeloablative regimens, non-myeloablative or reduced-intensity myeloablative conditioning regimens may be used in certain cases, and more particularly, for old patients or patients with comorbidities that are unable to tolerate this immunosuppressor treatment.<sup>11,14</sup> Moreover, an autograft after purging of malignant cells may constitute another alternative treatment in patients with high-risk leukemic relapse when no stem cell donor is available. For instance, the autologous transplantation of CD133<sup>+</sup> selected HSCs may be used for pediatric patients with relapsed CD34<sup>+</sup>/CD133<sup>-</sup> leukemia.<sup>42</sup> The results from a recent investigation have also revealed that the homing and engraftment of BCR-ABL<sup>+</sup> leukemic stem cells (LSCs) in the BM of patients with chronic myelogenous leukemias (CMLs) is highly dependent on CD44 adhesion molecule expression in respect to normal HSCs.<sup>46</sup> Therefore, the targeting of CD44 LSC using an anti-CD44 antibody also may constitute an alternative approach for improving the efficacy of HSC transplantation in CML patients.<sup>46</sup> In addition, bank stored-umbilical cord (UC) cells, including umbilical cord blood (UCB), placenta cells and fetal tissue-derived HSC transplants, which generally induce a less intense detrimental alloreactive response, may also constitute other HSC sources for autograft or allograft in certain clinical or experimental settings.<sup>9-11</sup>

### **Mesenchymal Stem Cells and Endothelial Progenitor Cells and Their Therapeutic Applications**

The BM stroma, PB, skin dermis and placenta as well as the walls of large and small blood vessels in most tissues and organs, including the brain, spleen, liver, kidney, lung, muscle, adipose tissues, thymus, uterus and pancreas, also contain the multipotent mesenchymal stem cells (MSCs) and endothelial progenitor cells (EPCs).<sup>9-11,47,48</sup> Much of the work conducted on adult stem/progenitor cells has focused on MSCs found within the BM stroma. More particularly, the MSCs expressing CD49a and CD133 markers are localized in a perivascular niche in BM, and may give rise to the osteoblasts that are colocalized with HSCs, and which may support the hematopoiesis by producing the growth factors and cytokines that promote the expansion and/or differentiation of HSCs (Fig. 1).<sup>1,2,5,9-11,49</sup> It has also been reported that the lung-resident mice CD45 side subpopulation containing MSCs and expressing a high telomerase level and mesenchymal markers (CD44, CD90, CD105, CD106, CD73, Sca-1) can differentiate into chondrocytes, adipocytes and osteocytes.<sup>50</sup> The results from differentiation studies have also indicated that CD146<sup>+</sup> Notch3<sup>+</sup> cells sorted from cultured BM-derived MSCs were capable of adipogenic and osteogenic differentiation, while ITGA11<sup>+</sup> cells mainly displayed an osteogenic differentiation profile with limited adipogenic fate.<sup>15</sup> The BM-derived or tissue-resident MSCs can generate diverse mesodermal cell lineages involved in osteogenesis, adipogenesis, cartilage and muscle formation, including the osteoblasts, osteocytes, adipocytes, chondrocytes, myoblasts and myocytes under appropriate culturing conditions *ex vivo* and *in vivo*.<sup>5,9-11</sup> Moreover, MSCs may also be induced to differentiate into fibroblasts, neuronal cells, pulmonary cells, pancreatic islet  $\beta$  cells, corneal epithelial cells and cardiomyocytes *ex vivo* and/or *in vivo* using specific growth factors and cytokines.<sup>5,9,10</sup>

In the case of EPCs, which are derived-like HSCs from the embryonic hemangioblasts, they may be distinguished by the expression of different biomarkers, including CD34<sup>+</sup> or CD34<sup>-</sup>, CD133, VEGFR2<sup>+</sup> also designated as Flk-1 (fetal liver kinase-1), KIT and CXC chemokine receptor 4 (CXCR4).<sup>14</sup> EPCs may contribute in a significant manner to give rise to mature endothelial cells that form new vascular walls of vessels after intense injury and vascular diseases as well as the new vessel formation in tumors.<sup>9-11</sup> The critical role of circulating EPCs in endothelial cell maintenance after tissue injury is notably supported by the observation that their number and function is inversely associated with the progression of atherosclerosis and an enhanced risk of cardiovascular diseases. For instance, it has been proposed that the number of circulating EPCs may be increased by down-regulating EPC senescence through plasma high-density lipoprotein (HDL)-induced nitric oxide (NO) production and telomerase activity via the phosphatidylinositol 3' kinase (PI3K)/Akt signaling pathway.<sup>22</sup> These molecular events may promote the angiogenic process, and thereby decrease the incidence of atherosclerosis-related ischemic diseases.<sup>22</sup> Moreover, the CXCR4 gene transfer in EPCs has been observed to promote their migration and adhesion to the endothelial cell layer and vascular re-endothelialization in nude mice subjected to carotid artery injury as compared to non-transduced EPCs, while a treatment with neutralizing antibodies against CXCR4 or/and JAK-2 inhibitor AG490 attenuated these beneficial effects.<sup>17</sup> In the same way, the transplantation of CXCR4-expressing EPCs also resulted in their migration to ischemic brain regions and promoted neurovascular repair, and improved long-term neurobehavioral outcomes in an animal model.<sup>23</sup> It has been noticed that an up-regulated expression of stromal-derived factor-1 (SDF-1) level in ischemic heart or brain can contribute in part to the recruitment of CXCR4-expressing EPC cells at injured tissues.<sup>16,23</sup>

All of the aforementioned functional properties of MSCs and EPCs made them the promising sources of immature cells for treating numerous degenerative and vascular disorders in human. The autologous or allogeneic transplantation of a BM or PB sample can lead to the homing and engraftment of functional HSCs, MSCs and EPCs and/or their differentiated progenies at the BM and distant damaged tissues. Thus, this supports the feasibility of this strategy for improving the tissue remodeling and healing processes after severe injury as well as in the treatment of diverse human disorders, including osteogenesis imperfecta, atherosclerotic lesions, visual loss associated with choroidal neovascularization, ischemic cardiovascular and muscular diseases.<sup>9-11,24,25</sup> It has been reported that MSCs, EPCs and their progenies can contribute to the vasculogenesis and regenerative process of several tissues, including bone, cartilage, tendon, muscle, adipose, brain, heart, lung, skin, pancreas, kidney and eye.<sup>10,24,25</sup> Importantly, adult BM-derived and tissue-resident MSCs are a little immunogenic and display immunomodulatory and anti-inflammatory effects in host *in vivo*.<sup>19</sup> In this regard, the results from a clinical trial consisting of a transplantation of autologous BM-derived MSCs to 41 patients between January 1998 and November 2008 have revealed that neither tumors nor infections were observed between 5 and 137 months of follow-up after transplantation.<sup>25</sup> Therefore, these therapeutic properties of MSCs also support their potential clinical applications to prevent the tissue or organ allograft rejection and severe acute and chronic GVHDs as well as to treat the autoimmune disorders such as inflammatory bowel diseases and inflammation of the heart muscle walls associated with autoimmune myocarditis, in which immunomodulation and tissue repair are required.<sup>19</sup> Indeed, MSCs can prolong skin allograft survival and reverse severe acute GVHDs *in vivo* supporting their use in treating skin diseases as well as in the maxillofacial surgery.<sup>51,52</sup> In counterpart, the



migration and proliferation of vascular smooth muscle cells (SMCs) derived from BM cells, including HSCs and MSCs, in the vascular injured area leading to an excessive cell accumulation, may contribute to the development of vascular pathologies such as intimal hyperplasia and atherosclerotic lesions.<sup>10,53</sup> Therefore, future investigations are necessary to optimize the BM-derived cell transplantation strategies and establish the specific mechanism(s) of action and physiological effects of HSCs, MSCs and EPCs in the long-term. This should allow for improvement of their therapeutic and curative benefits and prevent their detrimental clinical effects in treated patients.

## **CARDIAC STEM/PROGENITOR CELLS AND THEIR THERAPEUTIC APPLICATIONS**

The myocardial regeneration and cardiac function recovery in physiological and pathological conditions may occur via the activation of a small pool of interstitial cardiac stem/progenitor cells (CSCs or CPCs) found within the specialized niches localized at the apex and atria of the heart (Fig. 1).<sup>3,4,9-11</sup> Mammalian heart-resident adult CSCs or their early progenies expressing different stem cell-like markers, including telomerase, nestin, KIT (also designated CD117), multidrug resistance-1 (MDR-1) and ABCG2 multidrug transporters, Islet1 transcription factor and/or Sca-1 (in mice), are endowed with a high self-renewal capacity and multilineage differentiation potential.<sup>3,4,9-11</sup> These immature cells are able to give rise to three major cell types constituting the myocardium, including cardiomyocytes, smooth muscles and vascular endothelial cells in homeostatic conditions and after myocardial injuries.<sup>3,4,9-11</sup> Therefore, the *in vivo* stimulation of CSCs and early progenies by administration of diverse exogenous growth factors or the intravascular, intramyocardial or catheter-based delivery of *ex vivo* expanded CSCs or their differentiated progenies may represent the potential therapeutic strategies to treat and even cure diverse heart diseases (Fig. 1).<sup>3,9,10</sup> Moreover, the transplantation of genetically-modified adult stem/progenitor cells also offers great promise by permitting delivery of a specific therapeutic gene product such as an angiogenic agent or cardioprotective factor in the ischemic or non-ischemic heart disease areas (Fig. 1).<sup>10,26</sup> These treatment types, alone or in combination with the conventional clinical therapies by using pharmacological agents such as angiotensin-converting enzyme (ACE) inhibitors,  $\beta$ -adrenergic blockers, and nitroglycerin, represent promising strategies to improve the long-term survival of patients diagnosed with heart failures resulting from ischemic heart disease, hypertension and myocardial infarction.<sup>3,9-11,26,54</sup>

In addition, the use of other stem/progenitor cell types such as ESCs, UCB-derived stem cells (CD133<sup>+</sup> cells, HSCs or MSCs), alveolar epithelial stem cells (AECs), BM-derived stem cells (CD133<sup>+</sup> cells, HSCs, MSCs or EPCs), adipose-derived stem cells (ADSCs), muscle-derived stem cells (MDSCs), pancreatic stem cells (PSCs) and adult testicular stem cells or their progenies, which can differentiate into functional and contractile cardiomyocytes and/or vascular endothelial cells *in vitro* and/or *in vivo* also represent potential therapeutic stem/progenitor cell sources.<sup>3,10,16,17,26,27,55</sup> Consistent with this, the results from numerous investigations carried out on animal injury models *in vivo* have revealed the potential benefit of using these stem/progenitor cell types or their differentiated progenies with the cardiomyogenic properties to repair the damaged myocardium and improving the coronary revascularisation and cardiac function.<sup>10,11,21,26,27,55</sup> For instance, the data from small clinical trials consisting of the

transplantation of human BM-derived stem cells, mobilized PB cells or purified CD133<sup>+</sup> BM-derived stem cells into patients with advanced ischemic heart diseases have also indicated that this treatment generally improves the vascularization process and/or myocardial function.<sup>56-58</sup> Importantly, the results from a recent study have indicated that transendocardial injections of BM-derived MSCs also can improve the cardiac function by stimulating host heart-resident KIT<sup>+</sup> CSCs proliferation in an animal model of myocardial infraction.<sup>27</sup>

An optimization of cell delivery methods and cell-replacement therapies are required to establish the beneficial effects of these treatments on the ischemic and non-ischemic cardiac disorders versus their potential risk before they deemed safe to use in the clinical setting.

## NEURAL STEM/PROGENITOR CELLS AND THEIR THERAPEUTIC APPLICATIONS

### Phenotypic and Functional Properties of Neural Stem/Progenitor Cells

Adult neurogenesis and tissue repair in central and peripheral nervous tissues may occur through the activation of adult neural stem and progenitor cells (NSCs and NPCs).<sup>10,59</sup> The neural stem/progenitor cells have been identified within two specific germinal regions of the brain, the subventricular zone bordering lateral ventricle in the forebrain and dentate gyrus in hippocampus (Fig. 1).<sup>6-11</sup> Multipotent NSCs localized in the germinal subventricular zone, which express different stem cell-like markers, such as CD133 and nestin and possess a high self-renewal potential, can give rise to three principal cell lineages, including mature neurons and glial cells, astrocytes and oligodendrocytes.<sup>6-11</sup> NSCs can generate the progenitor cells that migrate along the blood vessels at distant damaged areas of the brain and participate to regenerate and repair the injured tissues by generating further differentiated and functional progenies.<sup>60</sup> Moreover, NPCs found in the subgranular cell layer of hippocampus, which are also designated neural precursor cells, can generate the granule cell projection neurons that integrate into existing neuronal circuitry.<sup>6-9,11</sup> In addition, multipotent adult stem/progenitor cells expressing the glial markers that are able to give rise to the dopaminergic glomus cells have also been identified in the peripheral nervous system within a germinal center termed carotid body.<sup>61</sup> Importantly, recent accumulating lines of experimental evidence have also indicated the possibility of using these different immature neural stem/progenitor cells in transplantation therapies for treating diverse neurological disorders.

### Potential Stem Cell-Based Therapies for Neurological Disorders

The discovery that the adult neural stem/progenitor cells can actively contribute to neurogenesis, astrogliogenesis and tissue repair in central and peripheral nervous systems in the postnatal developing brain and throughout adult life has given new avenues to develop novel stem cell-based therapies for treating diverse neurodegenerative disorders, cerebrovascular dysfunctions and primary brain tumors.<sup>10,59,62</sup> More specifically, the transplantation of neural stem/progenitor cells and/or oligodendrocyte precursor cells (OPCs) or other adult stem/progenitor cell types may constitute a potential cell-replacement strategy for treating diverse severe brain injuries and devastating



neurodegenerative diseases that are associated with a partial or substantial loss of functional neurons and/or glial cells. These neurological disorders include traumatic brain injury, Parkinson's and Alzheimer's, Lou Gehrig's and Huntington's diseases, temporal lobe epilepsy, stroke/cerebral ischemia, cerebellar ataxia, multiple sclerosis and amyotrophic lateral sclerosis.<sup>7,9-11,59,62-69</sup> It has been shown that ex vivo expanded neural stem/progenitor cells may be transplanted in specific brain regions where they can proliferate, survive, migrate at damaged areas and differentiate into functional neuronal and glial cells in vivo.<sup>7,9-11,59,62-69</sup> For instance, the intracerebral transplantation of neural stem/progenitor cells has been observed to improve neuronal survival under conditions of focal cerebral ischemia via the hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ )-induced VEGF expression.<sup>69</sup> Interestingly, it has also been observed that the neural progenitor cells from the olfactory organ of patients with Parkinson's disease were able to generate dopaminergic cells in vitro and reduce the behavioral asymmetry resulting from the dopaminergic neuron loss in the rat model of Parkinson's disease.<sup>67</sup> In regard with this, the intrastriatal transplantation of CB-stem/progenitor cells or their progenies also might constitute a potential cell source for anti-Parkinsonian therapy.<sup>61</sup> In the same way, the intravenous injection of human NSCs also resulted in their migration to the striatal lesion, where they attenuated the striatal atrophy and induced a long-term functional improvement in an animal model mimicking the striatal degeneration observed in Huntington's disease.<sup>63</sup>

In addition, ESCs, fetal stem/progenitor cells, umbilical cord-derived stem cells and other adult stem cell types, such as AECs, BM-derived MSCs, ADSCs and skin-, tooth- and endometrium-derived stem cells, may also be induced to differentiate or trans-differentiate into functional neurons, astrocytes or oligodendrocytes in vitro and/or in vivo.<sup>10,28-30</sup> For example, it has been observed that the transplantation of embryonic medial ganglionic eminence (MGE) cells into the striatum ameliorated motor symptoms in a rodent model of Parkinson's disease.<sup>28</sup> Moreover, the data from a small clinical trial with seven patients with Parkinson's disease treated with a single-dose of unilateral autologous BM-MSCs transplanted into the sublateral ventricular zone have indicated that three patients exhibited a steady improvement of the symptoms.<sup>10,29</sup> Importantly, it has also been reported that the hippocampal NSC transplantation rescued the cognitive decline in the learning and memory observed in aged animal model of Alzheimer disease by increasing hippocampal synaptic density induced by brain-derived neurotrophic factor.<sup>30</sup> In this matter, recent studies have also underlined the importance of considering the intrinsic and extrinsic factors that modulate neural stem/progenitor cell behavior as well as the influence of their local microenvironment to design more effective stem cell-based transplantation therapies for treating the brain disorders.

### **Stem Cell Transplant Plus Modulators of Neural Stem/Progenitor Cell Behavior**

The ex vivo treatment or in vivo administration of trophic factors that can modulate the functional integration, proliferation, long-term survival, fate specification and/or migration of neural stem/progenitor cells and/or their local microenvironment, alone or in combination with stem cell transplant, might represent potential therapeutic strategies for treating specific neurological diseases.<sup>10,31-36</sup> In this regard, it has been reported that the stimulation of sonic hedgehog (SHH) cascade using exogenous SHH ligand or chemically-synthesized agonist (SAG) of smoothened (SMO) hedgehog coreceptor induced the proliferation of neuronal and glial precursors in vitro.<sup>34</sup> Moreover, the

intracerebroventricular administration of SHH or SAG to adult rats promoted the survival of newly generated neural cells in adult rat models.<sup>34</sup> Importantly, it has also been reported that the transplantation of neural stem/progenitor cells, chondroitinase ABC and growth factors, EGF, basic FGF and platelet-derived growth factor-AA (PDGF-AA) principally resulted in their differentiation in oligodendrocytes and synergistically promoted the functional repair of chronically injured spinal cord in an animal model.<sup>35</sup> In the same way, the MSCs transplanted into the neurogenic areas of the hippocampus of the young rodent brain also were able to survive, migrate at distinct sites of graft and generate new mature neurons synthesizing neurotransmitters.<sup>36</sup> In contrast, the implantation of MSCs into non-neurogenic areas of striatum was associated with a massive cell degeneration and no migration of cells was seen under these conditions.<sup>36</sup> These results suggest then that the MSC behavior may be influenced by the local microenvironment prevalent within the site of implantation. Consistently, the induction of neuronal differentiation of MSCs by increasing cyclic adenosine monophosphate in the culture medium before cell implantation has been observed to promote their differentiation *in vivo*.<sup>36</sup> In addition, another promising strategy to treat neurological disorders and primary brain cancers also includes the transplantation of genetically-engineered adult stem/progenitor cells.

### Genetically-Engineered Stem Cell-Based Transplantation Therapies

The transplantation of genetically-engineered *ex vivo* expanded NSCs or other adult stem/progenitor cell types, including BM-derived stem cells, alone or in combination therapies, has been shown to effectively restore diverse neurological deregulations or suppress the brain tumor growth.<sup>70-77</sup> In fact, NSCs and adult progenitor stem cells such as MSCs possess an inherent tropic property and typically migrate throughout the brain to reach the injured areas or primary brain tumor sites, and more specifically in the tumor hypoxia region. Hence, human NSCs may be used as a delivery vehicle for a specific release of therapeutic gene products at damaged brain regions or primary brain tumors, including medulloblastomas and GBMs. For instance, it has been shown that the transplantation of NSCs expressing HIF-1 $\alpha$ , which can act, in part, by promoting angiogenesis, improved behavioral recovery in a rat stroke model.<sup>75</sup> The transplantation of fetal-derived NSCs engineered to express interleukin-12 or tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) related apoptosis inducing ligand (TRAIL), has also been observed to result in their specific recruitment within intracranial glioma, and the release of therapeutic gene product concomitant with an inhibition of tumor growth.<sup>78,79</sup> Similarly, the co-injection of neural stem/progenitor cells expressing cyclophosphamide (CPA)-activating enzyme cytochrome p450 2B6 (CYP2B6), which catalyzes CPA prodrug transformation into membrane diffusible DNA-alkylating metabolites with GMB cells followed by a CPA administration, markedly impaired brain tumor growth.<sup>80</sup> It has also been noted that the intracerebrally transplantation of CPA gene-engineered neural stem/progenitor cells resulted in their migration through the brain parenchyma to the neoplastic site concomitant with a tumor growth inhibition through the activation of CPA.<sup>80</sup>

Overall, these observations support the therapeutic interest of using adult stem/progenitor cells, which are able to integrate and migrate through the brain and reach the damaged brain regions, alone or in combination with other therapeutic agents, for developing effective transplantation therapies to rescue damaged areas of the brain or specifically target the brain tumor cells.

## OTHER TISSUE-RESIDENT ADULT STEM/PROGENITOR CELL TYPES AND THEIR THERAPEUTIC IMPLICATIONS

Among the other tissues and organs harboring an adult stem/progenitor cell subpopulation, there are skin, liver, intestinal crypts and gastric glands, adipose tissues, muscles, eye and pancreas (Fig. 1).<sup>9,10,14</sup> It has been shown that the *in vivo* stimulation of these adult stem/progenitor cells and/or the replacement of their dysfunctional counterparts and/or their further differentiated progenies by functional cells, also may constitute potential therapeutic strategies for the treatment of numerous pathological disorders in humans.<sup>9,10,14</sup> Particularly, the adult stem/progenitor cell-based transplantation therapies could result in the restoration of the regeneration program in these tissues, and thereby prevent the progressive loss of functions of these adult stem/progenitor cells with aging and lead to the treatment of diverse human disorders. Among the disorders that could be treated by adult stem/progenitor cell transplantations, there are skin disorders (chronic nonhealing wounds and ulcers, ectodermal dysplasia congenital disorders); lung disorders (interstitial lung diseases, cystic fibrosis, asthma, chronic bronchitis and emphysema); chronic liver injuries (hepatitis and liver cirrhosis); gastrointestinal disorders (chronic inflammatory bowel diseases and ulcers); bone and cartilage disorders (osteoporosis, osteogenesis imperfecta); musculoskeletal disorders (Duchenne and Becker dystrophies and amyotrophic lateral sclerosis); eye diseases (partial or total limbal and/or conjunctival stem cell deficiency, bullous keratopathy, glaucoma and retinal damages) and pancreatic disorders (Types 1 and 2 diabetes mellitus) (Fig. 1).<sup>9,10</sup> More particularly, the stimulation of PSCs *in vivo* or transplantation of *ex vivo* expanded pancreatic  $\beta$  cells in the host disease recipient may constitute a therapeutic strategy for restoring the  $\beta$  cell mass lost over time in diabetic patients.<sup>10,13,14</sup> Consistently, it has also been reported that the transplantation of purified pancreatic duct cells from islet-depleted human pancreatic tissue plus stromal cell preparation generated the insulin-producing cells in normoglycemic non-obese diabetes/severe combined immunodeficient (NOD/SCID) mice model *in vivo*.<sup>81</sup> Importantly, the gene therapy using insulin-producing cells, such as human adipose tissue-derived MSCs with unfractionated cultured BM, has also been observed to be effective for treating insulinopenic patients with Type 1 diabetes mellitus.<sup>82</sup>

In addition, it has been reported that the human endometrial gland-derived mesenchymal cells (EMCs) and menstrual blood-derived mesenchymal cells (MMCs) expressing CD29 and CD105 were more proliferative than MSCs from umbilical cord.<sup>83,84</sup> These pluripotent immature cells were able to differentiate into cardiomyocytic, respiratory epithelial, neurocytic, myocytic, endothelial, pancreatic, hepatic, adipocytic, and osteogenic cells *in vitro* as well as trans-differentiate into cardiac tissue-layer *in vivo*.<sup>83,84</sup> This suggests then that EMCs and MMCs may constitute new potential pluripotent stem cell sources, easily accessible for cell replacement-based therapy.<sup>83</sup>

On the other hand, the adult stem/progenitor cells engineered to express cytotoxic agents or antibodies targeting their malignant counterparts, cancer stem/progenitor cells and their local microenvironment also offer great promises for the development of new therapeutic approaches for treating aggressive, metastatic and recurrent cancers derived from these different tissue-resident adult stem/progenitor cells (Fig. 2).<sup>10,14,85,86</sup> As a matter of fact, it has been shown that the use of BM-derived EPCs engineered to express a soluble truncated form of VEGFR-2 impaired tumor growth *in vivo*.<sup>87</sup> MSCs engineered to produce and deliver tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) into tumor sites also caused the apoptotic death of lung (A549), breast (MDAMB231),

squamous (H357) and cervical (Hela) cancer cells in coculture experiments in vitro as well as a significant reduction of xenografted tumor growth and lung metastatic tumor burden in vivo.<sup>85</sup>

## CONCLUSION AND PERSPECTIVES

Together, these recent advancements in the field of the tissue-resident adult stem cell biology have led to the development of potential transplantation therapies for treating patients with diverse devastating diseases, including hematopoietic, cardiovascular and neurodegenerative diseases and aggressive and recurrent cancers.

Although important progress has been made, future studies are necessary to optimize the transplantation procedures in order to promote the functional integration, proliferation, differentiation and migration of transplanted adult stem stem/progenitor cells to damaged tissues and their long-term survival after implantation. Especially, the optimization of administration modes of tissue-resident adult stem/progenitor cell transplants and the identification of the specific intrinsic and extrinsic factors that regulate their behavior in physiological and pathological conditions is necessary for the design of new therapeutic strategies for improving the cell recovery and delivery in the specific damaged tissue areas after transplantation. These future studies should lead to more effective and safe transplantation therapies that could be translated in clinical settings for treating and even curing diverse human diseases which remain incurable in the clinics with the current conventional therapies.

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## MULTIPOTENT MESENCHYMAL STROMAL CELLS: Clinical Applications and Cancer Modeling

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**Abstract:** The recognition of the therapeutic potential of Multipotent Mesenchymal Stromal Cells (MSCs) is one of the most exciting recent advances in cell therapy. In just ten years, since the description of the multilineage potential of MSCs by Pittenger et al in 1999 until now, MSCs are being used in more than 150 clinical trials as therapeutic agents. The potential of these cells for cell-based therapies relies on several key properties: (1) their capacity to differentiate into several cell lineages; (2) their lack of immunogenicity and their immunomodulatory properties; (3) their ex vivo expansion potential; (4) their ability to secrete soluble factors which regulate crucial biological functions such as proliferation and differentiation over a broad spectrum of target cells; and (5) their ability to home to damaged tissues and tumor sites. Based on these properties MSCs are being exploited worldwide for a wide range of potential clinical applications including cell replacement strategies, treatment of graft-versus-host disease, autoimmune diseases and rejection after solid organ transplantation as well as their use as vehicles to deliver anti-cancer therapies. Importantly, the low inherent immunogenicity of MSCs means that they could be used not only for autologous but also for allogeneic cell therapies. In addition, increasing evidence has revealed a complex relationship between MSCs and cancer. Thus, solid evidence has placed MSCs transformed with specific mutations as the most likely cell of origin for certain sarcomas, and MSCs have been reported to both, inhibit or promote tumor growth depending on yet undefined conditions. Here we will thoroughly discuss the different potential clinical applications of MSC as well as the role of MSCs on sarcomagenesis and the control of tumor growth.

## INTRODUCTION

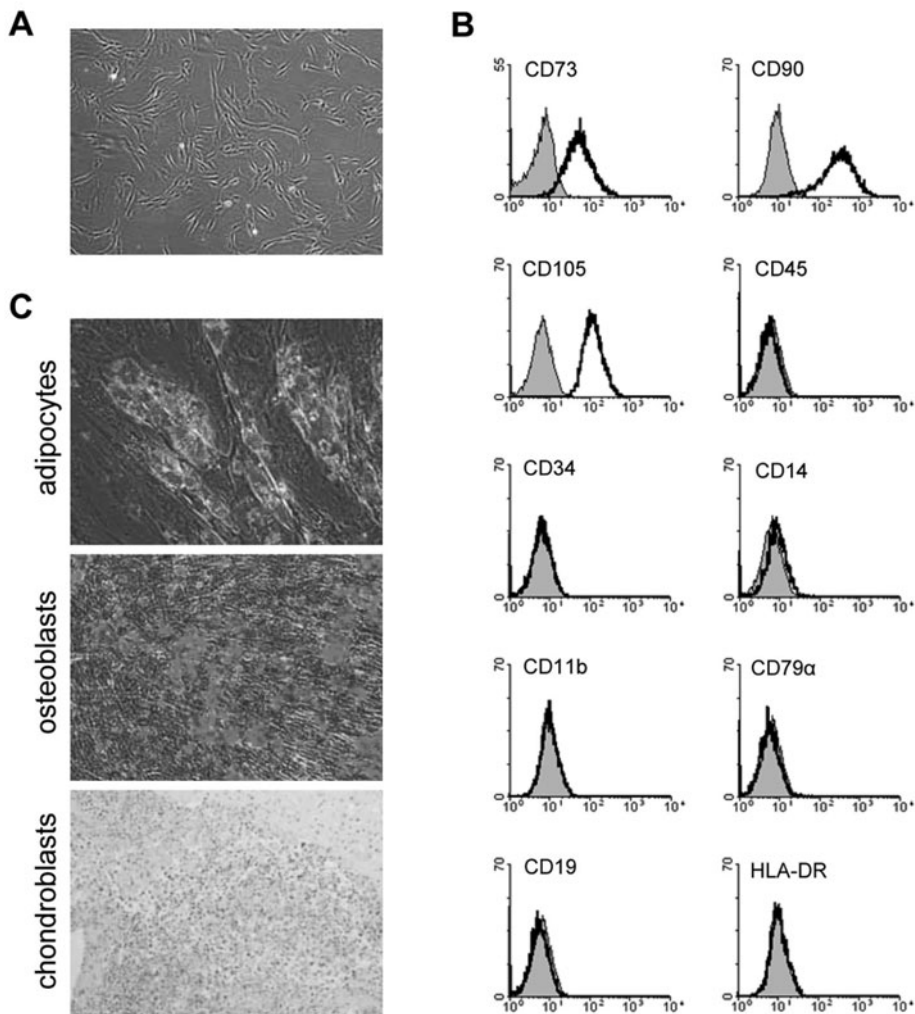
Pluripotent stem cells have been detected in multiple tissues in the adult, participating in normal tissue replacement and repair, while undergoing self-renewal. The best characterized source for adult stem cells is the bone marrow (BM). A subclass of bone marrow (BM) cells, commonly called Mesenchymal Stem Cells (MSC), can be isolated based on plastic-adherence properties and expanded in culture easily.<sup>1</sup> However, the recognized biological properties of the unfractionated population of MSCs do not seem to meet generally accepted criteria for stem cell activity. Following the guidelines of the International Society for Cellular Therapy (ISCT) the appropriate term for these precursors should be Multipotent Mesenchymal Stromal Cells, while the term mesenchymal stem cells should be used only for cells subpopulations that meet stem cell criteria. In any case, the widely recognized acronym, MSC, could be maintained.<sup>2</sup> Additionally, the ISCT proposes a minimal criteria to define these multipotent precursors: (1) MSCs must be plastic-adherent when maintained in standard culture conditions; (2) MSCs must express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79 $\alpha$  or CD19 and HLA-DR surface molecules (additional markers commonly used to characterize MSC cultures are summarized in Table 1) MSCs must differentiate to osteoblasts, adipocytes and chondroblasts in vitro<sup>3</sup> (Fig. 1). Cells fulfilling these properties are present in a variety of tissues during development and therefore can be isolated from several and perhaps most adult tissues including adipose tissue, umbilical cord, liver or muscle<sup>4,5</sup> although

**Table 1.** Common surface markers used to characterize human MSCs

Molecule	Alternative Name	Staining in MSCs
CD13	APN	+
CD14	LPS-R	–
CD15	Lewis Ag	–
CD29	Integrin $\beta$ 1	+
CD31	PECAM-1	–
CD34	Sialomucin	–
CD44	Hyaluronate receptor	+
CD45	Leucocyte common antigen	–
CD73	Ecto 5' nucleotidase	+
CD79 $\alpha$	Immunoglobulin-associated alpha	–
CD80	B7-1	–
CD86	B7-2	–
CD90	Thy-1	+
CD105	Endoglin	+
CD117	c-KIT	–
CD133	AC133	–
CD144	Cadherin-5	–
CD146	MUC-18	+
CD164	MUC-24	+
CD166	ALCAM	+
MHC class I		+
MHC class II		–

BM represents the source most often used. The prevailing consensus is that MSCs have similar biological characteristics regardless of tissue source, but differences are likely to be reported in the near future.

Many open questions still remain about the main nature and identity of MSCs including origin, location and multipotency capacity: (1) it is now well accepted that MSCs constitute a source of progenitors of mesoderm-derived tissues such as bone,



**Figure 1.** Characterization of human MSCs according to the guidelines of the International Society for Cellular Therapy. A) Plastic-adherent cells showing fibroblast-like fusiform morphology. B) Flow cytometry analysis of the positive and negative markers that define MSCs. Filled lines represent the irrelevant isotypes. Empty lines display antibody-specific staining C) Differentiation of MSC cultures to adipocytes (Oil red-O staining), osteoblasts (Alizarin red staining), and chondroblasts (Alcian blue staining) after incubation of cells with specific differentiation inductive media.<sup>14</sup>

cartilage and fat, but MSC cultures are heterogeneous and different subpopulations have been reported to vary in their proliferative and multilineage potential;<sup>6</sup> (2) although most, if not all, common MSCs markers are highly modulated in culture, other markers such as STRO-1,<sup>7</sup> CD271,<sup>8</sup> SSEA-1/CD15,<sup>9</sup> SSEA-4,<sup>10</sup> CD146,<sup>11</sup> or GD2<sup>12</sup> have been proposed to identify an *in vivo* mesenchymal precursor, though it is not clear yet whether they define a subset of MSCs or the “master” very immature MSC; (3) in addition to the adipogenic, osteogenic and chondrogenic potential, MSCs have been reported to differentiate into other mesenchymal tissues such as skeletal muscle and nonmesenchymal tissues such as liver, brain or pancreas<sup>13-16</sup> although this ability to differentiate into nonmesenchymal cell lineages is tremendously controversial; and (4) the exact nature and localization of MSCs *in vivo* remain poorly understood but increasing evidence indicates that MSCs precursors from different tissues could have a perivascular distribution.<sup>17,18</sup>

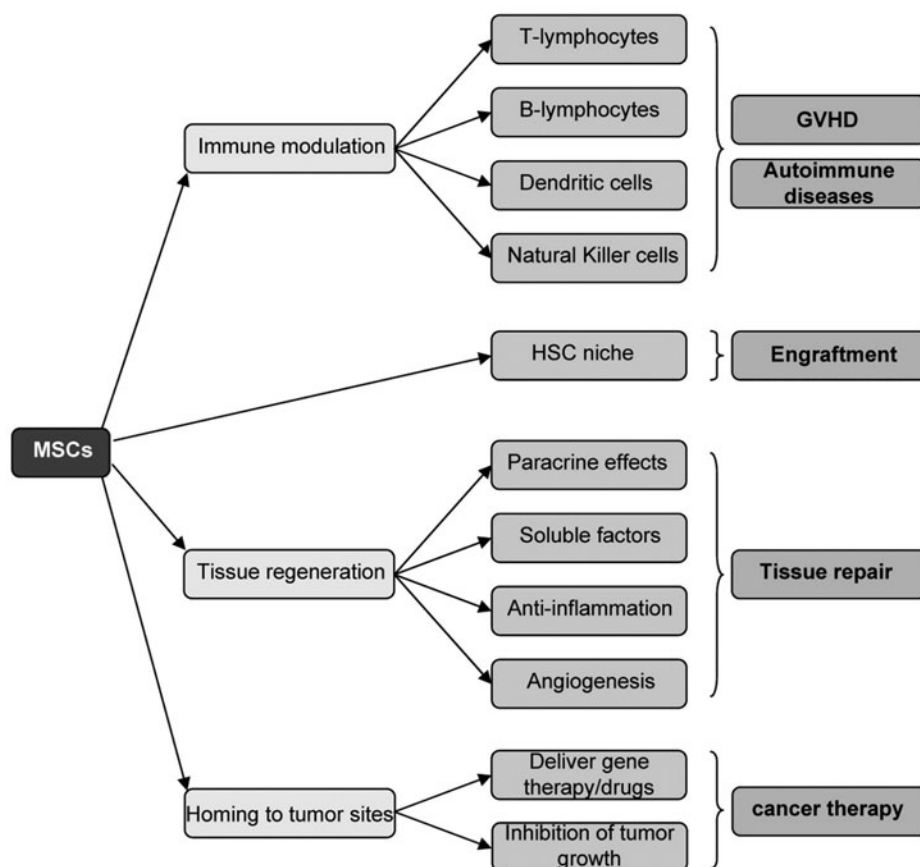
The recognition of the therapeutic potential of MSCs is likely one of the most exciting recent advances in cell therapy. The potential clinical use of MSCs in tissue repair mainly covers bone, cartilage and tendon. Proof-of-principle for MSC-based cell therapy has already been established for bone as discussed below, however, whether MSCs can generate any other tissue *in vivo* still remains to be experimentally and clinically elucidated.

Apart from tissue repair, an emerging body of evidences shows that MSCs possess additional properties that could be beneficial in many disease processes including their ability to enhance the engraftment after hematopoietic stem cell (HSC) transplantation (HSCT) and their immunomodulatory features. MSCs are part of the BM microenvironment supporting the survival, growth and differentiation of HSCs. Although recipient MSCs mostly survive to myeloablative conditioning regimen and post-transplant MSCs are predominantly recipient-derived,<sup>19-21</sup> cotransplantation of expanded MSCs results in faster HSC engraftment and nearly full donor chimerism.<sup>22</sup> Likewise, MSC's role in modulating the immune response is now attracting the most interest as they are being tested in numerous trials for a variety of nonhaematological indications<sup>23</sup> ([www.clinicaltrial.gov](http://www.clinicaltrial.gov)).

Furthermore, several studies have shown that MSCs are not inherently immunogenic and therefore escape from immune surveillance *in vivo*,<sup>24-26</sup> which means that MSCs could be used not only for autologous but also for allogeneic cell therapies. Despite being costly, the establishment of a stem-cell bank of well characterized and “ready-to-use” allogeneic MSCs under GMP (Good Manufacturing Practice) conditions could be an interesting approach towards facilitating the access of investigators and clinicians to this valuable material.

*Ex vivo* expansion of human MSCs (hMSCs) is a prerequisite for the use of these cells in cell-based therapies. MSCs have different control points, which regulate their lifespan *in vitro*, resulting in an *in vitro* limited cell growth. Accordingly, there is certain controversy as to whether MSCs undergo *in vitro* tumoral transformation or genetic instability.<sup>27,28</sup> It is worth mentioning that the use of MSCs for clinical purposes will require the biosafety of these primary cells to be carefully investigated through appropriate and sensitive cellular, molecular and genetic tests.

Finally, increasing evidence has revealed a complex relationship between MSCs and cancer. Thus, solid evidences have placed MSCs transformed with specific mutations as the most likely cell of origin for certain sarcomas. Likewise, MSCs have been reported to both, inhibit or promote tumor growth depending on yet undefined conditions. Furthermore, the ability of MSCs to home to tumor sites has placed these cells among the most promising vectors for many types of antitumor therapies.



**Figure 2.** Scheme showing the most relevant properties of MSCs, their therapeutic effects and targets and their potential clinical applications. Adapted from Battiwalla M, Hematti P. *Cytotherapy* 2009; 11:503-515<sup>38</sup> with permission of The International Society for Cellular Therapy.

In this chapter we will deeply discuss the different potential clinical applications of MSCs (Fig. 2) as well as the complex relationship between MSCs and cancer.

## CLINICAL APPLICATIONS BASED ON MSCs: OVERVIEW OF ONGOING CLINICAL TRIALS

Over the last decade, our understanding and the clinical applications of MSCs have developed rapidly. The potential of these cells for cell-based therapies relies on several key properties: (1) their capacity to differentiate into several cell lineages; (2) their lack of immunogenicity and their immunomodulatory properties; (3) their *ex vivo* expansion potential; (4) their ability to secrete soluble factors which regulate crucial biological functions such as proliferation and differentiation over a broad spectrum of target cells; and (5) their ability to home to damaged tissues and tumor sites.

Much has been learned about MSCs and much remains to be learned. However, their immunomodulatory properties, their paracrine interactions with specific cell types in damaged tissues and the promising results achieved in some clinical applications have already made these cells an attractive option for the treatment of various diseases. MSCs possess immunological properties that have been demonstrated both *in vitro* and *in vivo*. Although the exact mechanisms underlying these effects remain largely unknown, new insights have been gained over the last years. MSCs preferentially home to damaged tissues and secrete paracrine anti-inflammatory factors. The immunomodulatory, reparative and anti-inflammatory properties of MSCs have been tested in a variety of animal models and are being applied in certain specific clinical settings.

An interesting opportunity offered by the MSC is the possibility of using allogeneic cells. First, MSCs are non-immunogenic due to the lack of expression, before differentiation, of MHC molecules (class II) on their surface.<sup>29</sup> Second, the ability of allogeneic MSCs to inhibit the generation of mature dendritic cells (DCs) and the activation of T cells has been demonstrated. The differentiation of mature DCs is interfered by MSCs by inducing the appearance of immature DCs which exhibit an inhibitory phenotype.<sup>30</sup> This phenomenon is partially associated with the synthesis of IL6, PGE-2 or M-CSF.<sup>31,32</sup> MSCs may potentially induce T-cell anergy due to the lack of ligands for CD28 and other costimulatory molecules on their membrane leading to a state of immunological ignorance.<sup>29</sup>

From a clinical standpoint, MSCs are considered to be drugs and therefore need to follow the same legal manufacturing requirements (GMP) if they are to be used in clinical practice.<sup>33</sup> This point has been crucial for the development of many clinical applications. The potential clinical applications of MSCs include prevention and treatment of therapy-resistant graft-versus-host disease (GVHD), prevention and treatment of rejection after solid organ transplantation, tissue repair, treatment of inflammatory and autoimmune diseases as well as the use of MSCs as vectors to deliver anti-cancer therapy. Finally, we should not ignore the attempts being made to achieve organ regeneration.

## **TREATMENT OF GRAFT-VERSUS-HOST DISEASE**

Exploiting the immunomodulatory capacity of MSCs, they can be used to facilitate allogeneic cell replacement therapies regardless of the degree of HLA match between donor and recipient. Allogeneic HSCT is the therapy of choice for many hematologic malignancies. The immune effect of the T-lymphocytes present in the graft may recognize and exert a cytotoxic effect against tumor cells from the host, inducing a graft-versus-leukemia (GVL) effect. One of its main complications is GVHD, caused by the donor's T-lymphocytes reacting against recipient's antigens. If Grade II or higher GVHD occurs despite conventional GVHD prophylaxis, steroids are able to control these side effects in about half of the patients. However, steroid-resistant GVHD is associated to very poor prognosis and the chances of overall survival are of less than 10%.<sup>34-36</sup> In these cases MSCs from various origins have been used in several different clinical trials. Specifically, Le Blanc et al<sup>26</sup> transplanted haploidentical MSCs into patients with severe treatment-resistant Grades III-IV acute GVHD. The acute GVHD disappeared completely in 75% of patients,<sup>37</sup> and currently more than 127 patients are enrolled on this study. Other clinical groups, spurred on by these results, have initiated twenty clinical trials to treat GVHD. Today, this is the most advanced application of MSCs. The largest clinical trial focused on this pathology has been set up by Osiris Therapeutics, Inc. (September,



2009). The preliminary results from their Phase III clinical trial treating severe GVHD with allogeneic MSCs infused either as first line treatment or in combination with steroids have been published in his webpage ([www.osiristx.com](http://www.osiristx.com)). The endpoint of this study was the proportion of patients surviving at least 90 days and achieving a complete response when allogeneic MSCs were added to steroid therapy, compared to those receiving only steroids. In another trial, allogeneic MSCs were infused to patients who did not respond to corticosteroids, with the primary endpoint being a durable complete response for a period of at least 28 days ([www.clinicaltrial.gov](http://www.clinicaltrial.gov)). In summary, most of the experimental and clinical experiences with MSCs for the treatment of severe GVHD points to a survival improvement. These results were similar for MSCs from different sources, distinct degrees of HLA compatibility and family relationships. However, many aspects remain to be clarified, such as the effect of the combination of MSCs with other immunosuppressive therapies or the selection of particular GVHD clinical settings to obtain better response rates. Additionally, particularly regarding the preventive use of MSCs in GVHD, the impact of their immunosuppressive properties on the beneficial Graft-versus-Leukemia effect, has to be clarified.

Importantly, in addition to their immunosuppressive effect, MSCs enhance hematopoietic engraftment and data from several clinical trials confirm that MSCs reduce the risk of graft failure in patients undergoing a haploidentical HSCT.<sup>22,38</sup>

## TREATMENT OF AUTOIMMUNE DISEASES

The effects of MSCs on autoimmune processes have been studied in a few experimental pre-clinical models. Several studies reported beneficial effects of MSCs. Gerdoni et al<sup>39</sup> observed the improvement of autoimmune encephalomyelitis, an experimental model for human multiple sclerosis, in animals treated with syngeneic MSCs. Treated animals showed reduced demyelization and fewer inflammatory infiltrates. Currently, the mechanisms involved in the possible *in vivo* immunomodulatory effects of MSCs remain an unresolved issue. It is known that MSCs are influenced by immunocompetent cells through the expression of numerous receptors for cytokines, including IL1, IL3, IL4, IL6, IL7, IL15, IFN $\gamma$  and TNF $\alpha$ <sup>40</sup> and chemokines, such as CCR1, CCR7, CCR9, CXCR4, CXCR5, CXCR6,<sup>33</sup> as well as TLR2 and other Toll-like receptors.<sup>41</sup> The responses of MSCs to the signaling through these receptors not only affect their survival, proliferation and differentiation capacities, but also the profile of soluble factors produced by MSCs that can then affect the immune system. Numerous clinical trials are being carried out taking advantage of the proven anti-inflammatory and immunomodulatory capacity of MSCs. In particular, it is important to mention the use of MSCs from different sources to treat diseases of the nervous system, such as multiple sclerosis; orthopedic problems, such as rheumatoid arthritis; pulmonary diseases, such as asthma; and chronic diseases of solid organs, such as chronic pancreatitis. In this area, we should highlight the clinical trials in multiple sclerosis and rheumatoid arthritis as the most advanced and those involving larger cohorts. Worth mentioning, the treatment of Crohn's could be one of the most promising applications of MSCs. There are currently three Phase III trials, two with allogeneic MSCs and one with autologous MSCs. However, all of them were stopped in March 2009 because an interim analysis showed an excessive response in the placebo group as reported by Osiris Therapeutics Inc in a press release. The press release clarifies that stopping recruitment was not due to any safety concerns. These results were commented in a recent report.<sup>42</sup>



## **PREVENTION AND TREATMENT OF REJECTION AFTER SOLID ORGAN TRANSPLANTATION**

As aforementioned, many clinical groups have explored the immunomodulatory capacity of MSCs. In most cases, MSCs have been used to prevent rejection of transplanted organs, especially cotransplantation with pancreatic islets, kidney, heart and liver. Currently, more than 12 clinical trials are being conducted where MSCs from the donor are cotransplanted before liver or kidney transplantation. The results are very promising and call for an increase in the number of trials aimed at avoiding rejection after solid organ transplantation. This may be an important use of MSCs in the near future.

## **USE OF MSCs IN TISSUE REPAIR**

There is a great deal of scientific and clinical interest in the potential of MSCs to stimulate wound repair. MSC-based therapies represent a new type of treatment for preventing morbidity and disability from chronic wounds, an unresolved clinical problem in which there has been little progress in recent decades.<sup>43</sup> The healing process requires a well-orchestrated integration of complex biological and molecular events. However, this orderly progression is impaired in many chronic diseases.<sup>44</sup> Functional characteristics of MSCs such as their ability to migrate to the site of injury or inflammation to stimulate proliferation and differentiation of resident progenitor cells through growth factor secretion and matrix remodeling, and their immunomodulatory and anti-inflammatory effects, may benefit wound healing. Recent studies have demonstrated that treatment with BM-MSCs accelerates wound healing kinetics, increases epithelialization and angiogenesis,<sup>45-47</sup> suggesting that MSCs enhance wound repair by at least two different mechanisms: differentiation and paracrine interactions with specific cell types in cutaneous wound.<sup>48,49</sup> The application of MSC therapy to human wounds has shown excellent results as described in recent studies. In the last three years the number of trials for digestive diseases has increased 10 fold, while major burns<sup>50</sup> and perianal fistulas<sup>51</sup> are the two main diseases for which stem cell therapy is being tested. Stem cell applications for most of these indications are currently at the stage of clinical development. The most advanced clinical trial has recently been completed for the treatment of complex perianal fistulas (Phase III). Although the results have not yet been released by the sponsor (Cellerix), it is known that the fistula tract closed in a high percentage of patients treated in a shorter period and, perhaps most importantly, no adverse effects have come to light. Indeed, it should be emphasized that allogeneic MSCs have been applied in various clinical trials involved in tissue repair without adverse effects ([www.clinicaltrials.gov](http://www.clinicaltrials.gov)).

## **ADVANCES IN ORGAN REGENERATION BY MSCs**

The regeneration of tissues and organs has long been the dream of most developmental biologists and stem cell researchers. MSCs have opened important lines of research. Due to its mesodermal origin, tissue regeneration of bone and cartilage are the areas that have most benefited. Among them, the bone is probably at the forefront of current cell replacement and tissue repair applications. This may be, at least in part, due to the better understanding of the cellular and molecular mechanisms implicated in MSC-mediated osteogenesis. Proof-of-principle for MSC-based cell therapy for bone repair has already been established, as MSCs are currently being exploited to repair segmental bone defects

of critical size in animals,<sup>52</sup> to restore healing of non-union long bone fractures in humans (www.aastrom.com) or to treat bones of children with osteogenesis imperfecta.<sup>53</sup> Human clinical trials addressing tissue engineered grafts in bone repair are limited only to some case reports.<sup>54,55</sup> The autologous BM-MSCs were loaded on ceramic, slowly absorbable scaffolds. The results confirmed safety of the products and demonstrated integration of the constructs into the bone. In another report from Gangji et al, two patients with osteonecrosis of the femoral head were treated by injection of BM-MSCs.<sup>56</sup> Osteoprogenitors and osteoblasts from BM were separated and expanded *in vitro*, and injected into the necrotic zone after differentiation under autologous conditions. Pain reduction, necrotic lesion decrease and functional improvement were recorded in the early period, and only minor side-effects were reported. Although these preliminary reports are poorly controlled and need further confirmation, the early signs are encouraging.

In addition to the osteogenic differentiation capacity and their potential in bone regeneration, MSCs might also be used for restoration of function in individuals suffering from cartilage damage. The cell collection procedure from BM is much less invasive than that required for autologous cartilage implantation (ACI), and can be performed under local anesthesia in the outpatient clinic. This also means that surgery is required only once, while ACI requires two surgeries. Furthermore, in the case of ACI, cell harvesting results in damage of the normal articular surface and the proliferation capacity of MSC is greater than that of chondrocytes. Additionally, MSCs can be harvested without impairing normal articular cartilage and displays high proliferation as well as chondrogenic differentiation capacity. Interestingly, autologous MSCs have been used dispersed in gels of collagen-Type-I or alginate in order to enhance the chondrogenic differentiation for repairing defects on femoral condyles.<sup>57</sup>

Myogenic differentiation of MSCs is currently being applied to cardiac and skeletal muscle regeneration research.<sup>58,59</sup> Recent reviews have summarized the effects of clinical trials in which satellite cells (SC, the stem cell of skeletal muscle) and BM-MSCs were transplanted to ameliorate the effects of myocardial infarction.<sup>60-62</sup> The results have been generally disappointing, with improvements in cardiac function ranging from non-existent to modest. Neither SCs nor BM-MSCs differentiated into new cardiomyocytes. The SCs differentiated into skeletal muscle that was not electrically coupled to host cardiac muscle, but may have augmented contraction of the heart muscle. BM-MSCs might have had a positive effect in two ways, through endothelial progenitors contributing to new microvasculature, and by paracrine/juxtacrine effects on the survival of host cardiomyocytes.

## USE OF MSCs AS VEHICLES TO DELIVER ANTI-CANCER THERAPIES

Tumor cells induce the migration of MSCs to the tumor site through the secretion of specific factors.<sup>63</sup> For example, secretion of VEGF, FGF2<sup>64</sup> and CCL-2<sup>65</sup> from breast cancer cells, or SDF-1 from osteosarcoma,<sup>66</sup> recruits BM-derived hMSCs to the stroma of the tumors. This ability of MSCs to migrate and seed tumors has been exploited to specifically deliver different types of cancer therapies. Thus, MSCs have been successfully used as vehicles to efficiently deliver oncolytic viruses into tumors and metastatic sites in xenograft models of breast carcinoma,<sup>67</sup> ovarian cancer<sup>68</sup> and glioma.<sup>69,70</sup> Moreover, the efficiency of this type of therapy has also been proven in infant patients with metastatic neuroblastoma refractory to front-line therapies.<sup>71</sup> MSCs are also being used to deliver anticancer agents. Thus, MSCs over-expressing different cytokines (IFN- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$ , IL-2 and IL-12), chemokines (CX<sub>3</sub>CL1) or growth factors (NK4) have suppressed

tumor growth in different tumor and metastasis models.<sup>72</sup> Likewise, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been expressed in hMSCs derived from different tissues, and these TRAIL-producing MSCs have effectively inhibited the growth of different types of tumors by the selective induction of apoptosis in tumoral cells.<sup>73-78</sup> Finally, MSCs have also been used to deliver prodrug-converting enzymes like the herpes simplex virus-thymidine kinase (HSV-tk) into tumors, where HSV-tk converts the systemically administrated prodrug ganciclovir into its toxic form. This strategy has demonstrated significant antitumor effects in gliomas<sup>79</sup> and pancreatic cancer.<sup>80</sup> MSCs have also been used to deliver another prodrug-converting enzyme, cytosine deaminase, in combination with the prodrug 5-fluorocytosine, which is converted to 5-fluorouracil in tumors. This therapy has been proven in different experimental tumor models.<sup>81,82</sup>

CLINICAL REALITY

Based on the above described information, if we analyze the data available at ClinicalTrials.gov, we can see the significant progress that has been made in the number and diversity of clinical trials using MSCs (Table 2). Among the different cell sources three in particular should be highlighted: BM-MSCs, adipose tissue-derived MSCs (also know adipose derived adherent stem/stromal cells, ASC) and peripheral blood MSCs. Over the last two years, the use of MSCs from placenta and umbilical cord blood (CB) has increased. In addition to clinical trials, more than 6.000 patients treated under compassionate use as isolated clinical cases are described in the literature. For this system, patients with widely different conditions have been treated, from tracheal resections to Type I diabetes, as well as skin regeneration and bone reconstructions, both in adult and pediatric patients.

Unfortunately, cell therapy with MSCs is not yet a clinical reality. Though to date no clinical application has successfully passed a Phase III effectiveness trial (Table 2), we consider it only a matter of time. Already, it is indisputable that many patients have improved their condition and, most importantly, there have been good results on the safety of the use of MSCs and improved quality of life of patients in almost all trials.

The recognition of stem cells as a therapeutic agent along with the new legislation that has been drawn up will ensure that MSCs do arrive on the market (provided the Phase III studies confirm the promise shown so far) and that they are used safely and properly controlled.

**Table 2.** Current distribution of clinical trials using MSC (ClinicalTrials.gov)

Type of Study	Clinical Trials	Allogeneic/ Autologous	ASC	BM-MSC	CB-MSC	MSC in Gene Therapy <sup>§</sup>	Other MSC Origins*
Phase I	52	19/33	12	25	3	3	9
Phase II	72	24/48	9	35	6	1	21
Phase III	8	1/7	2	3	1	0	0
Phase IV	0	0/0	0	0	0	0	0
Cancer related	31	10/21	7	11	3	0	10

<sup>§</sup> Clinical trials using MSCs as vehicles to deliver gene therapy.

\* Clinical trials using scaffolds-associated MSCs, predifferentiated MSCs and MSC-derived cell types such as chondrocytes.

Autologous therapy can delay the stem cell procedure due to the time needed to sample and purify the cells and certain patients may be excluded. Thus, an increasing number of studies are being performed with allogeneic cells. If similar results were to be obtained, stem cell banks should be created.

On the other hand, simplification of cell expansion with closed systems, currently under development, will help make these therapies more accessible and less costly. The creation of international banks could facilitate access for developing countries. Basic research continues to try to obtain certain types of cells that are able to become integrated and functional within the organ forming part of the complex homeostasis of an organism. If this could be achieved, the jump from reparative to regenerative therapy could be attained. Today, this may sound like science fiction, but the results obtained recently would also have seemed as far-fetched just a few years ago.

## ROLE OF MSCs ON SARCOMAGENESIS AND TUMOR GROWTH

There are two proposed models for cancer genesis and development: The stochastic model and the hierarchical model. In the later, it is hypothesized that different cells within the tumor have distinct potential to initiate and maintain a tumor, existing a rare cellular fraction of cancer stem cells (CSC) capable of long-term tumor maintenance. These CSCs, which have been recently identified in many types of tumors, are the only cells in the malignancy with ability to expand and promote tumor growth.<sup>83</sup> Therefore, the accurate identification and characterization of these subsets of cells will provide great advances in the development of new and more efficient therapies which could probably avoid the tumor relapses observed after conventional chemotherapy treatments targeted against the bulk tumor but that usually does not affect dormant CSCs.

The CSC model also suggests that these CSC could be normal stem cells that acquired the genetic hits necessary for malignant transformation. In this regard, increasing evidences suggest that sarcomas could be good examples of the CSC model and that MSCs might be the target cell for the transforming mutations that give rise to these types of tumors. Thus, several types of human sarcomas have been reproduced *in vivo* upon the over-expression of specific fusion oncoproteins in BM-derived mouse MSCs (mMSCs, Table 3). These models include the recapitulation of Ewing's Sarcoma by expression of EWS-FLI-1,<sup>84,85</sup> Mixoid Liposarcoma (MLS) by expression of FUS-CHOP<sup>86</sup> and Alveolar Rhabdomyosarcoma by expression of PAX-FKHR.<sup>87,88</sup> In addition, CSCs displaying MSC properties have been recently identified in Ewing's Sarcoma.<sup>89</sup> In another work, the expression of EWS-FLI-1 fusion protein in hMSCs induced a pattern of gene expression similar to that observed in human Ewing's sarcoma although does not produce cell transformation.<sup>90</sup> Furthermore, the treatment of Ewing tumor cell lines with specific EWS-FLI1 short hairpin RNA shifted their gene expression profile towards that of MSCs.<sup>91</sup> The primary effect of a specific translocation seems to be the blockage of a particular differentiation process, thus, the degree of tumor differentiation is thought to depend on the stage of cell maturation at which the transformation occurs. In this regard it has been suggested a classification of liposarcomas (from dedifferentiated to well-differentiated liposarcomas) based on the adipogenic differentiation state of the MSCs that give rise to the tumors.<sup>92</sup> These results suggest that MSCs may provide an ideal environment for the initiation of some sarcomas upon the expression of specific translocations but also that secondary transforming hits are needed to fully transform hMSCs.

**Table 3.** Models of sarcoma derived from transformed MSCs

Sarcoma Type	Oncogenic Events	Mouse/Human	MSC Source	References
Ewing's sarcoma	EWS-FLI-1	Mouse	BM	84-85
Alveolar Rhabdomyosarcoma	PAX-FKHR + p53 inactivation	Mouse	BM	88
Mixoid Liposarcoma	FUS-CHOP	Mouse	BM	86
Osteosarcoma	p53 and Rb inactivation	Mouse	BM	104
Leiomyosarcoma	p53 and Rb inactivation	Mouse	Adipose tissue	107
Fibrous histiocytoma	Wnt signalling inactivation	Human	BM	97
No tumors—gene expression similar to Ewing's sarcoma	EWS-FLI-1	Human	BM	90

In addition to the expression of sarcoma-associated fusion genes, the alteration of relevant signaling pathways can also transform MSCs giving rise to the formation of different type of sarcomas (Table 3). In this regard, Wnt signaling seems to be important in tumorigenesis induced by MSCs. The canonical Wnt/ $\beta$ -catenin signaling pathway plays a central role in modulating the balance between self-renewal and differentiation in stem cells.<sup>93</sup> In addition, Wnt signaling also regulates the invasion capacity and the proliferation of hMSCs.<sup>94,95</sup> While these properties exerted by the Wnt pathway may be useful in tissue regeneration, an inadequate activation of this pathway may deregulate the balance between proliferation, differentiation and apoptosis leading to a malignant transformation.<sup>96</sup> Importantly, the inactivation of Wnt signaling leads to transformation of hMSCs and the formation of malignant fibrous histiocytoma (MFH) and, conversely, restoration of Wnt signaling in MFH cells allows them to differentiate along connective tissue lineage.<sup>97</sup> Furthermore, key components of the Wnt signaling pathway are downregulated in osteosarcoma compared to normal MSCs and MSCs differentiated to osteoblasts.<sup>98</sup>

The process of MSC transformation is frequently associated to the accumulation of chromosome instability.<sup>99-101</sup> This observation, together with the high resistance of MSCs to apoptosis,<sup>102,103</sup> suggests the relevance of an accurate cell cycle control in MSCs. Several reports have demonstrated the relevant role that the deficiency of different cell cycle regulator proteins, especially *p53*, has on the transformation of mMSCs (Table 3).<sup>104-107</sup> Importantly, the type of sarcomas obtained from *p53*-deficient MSCs seem to depend on the tissue of origin of the MSCs undergoing transformation. Thus, leiomyosarcoma would be linked with the loss of *p53* in fat-tissue derived MSCs<sup>107</sup> while the loss of this cell cycle regulator in BM-MSCs or their derived osteogenic lineage would result in osteosarcoma development.<sup>104</sup> Interestingly, *p53* mutations and *p53* deregulated expression are frequent in sarcomas<sup>108-111</sup> supporting *p53* as a potential secondary hit candidate for hMSC transformation. In this regard, it has been reported that *p53* knock-down is required as secondary hit to allow tumor formation in a model of Alveolar Rhabdomyosarcoma based on PAX-FKHR fusion oncoprotein expression in mMSCs.<sup>88</sup> The loss of other key

cell cycle regulator such as *Rb* did not transform MSCs but its deficiency potentiates tumor development from *p53*-deficient MSCs,<sup>104,107,112</sup> giving rise to less differentiated sarcomas.<sup>107</sup> Likewise, alterations in other cell cycle regulators such as p16<sup>INK4A</sup> or p19<sup>ARF</sup> have also been detected in transformed MSCs.<sup>106,113</sup> Furthermore, other common events associated to the transformation process include the overexpression of c-myc<sup>99,106</sup> and the acquisition of telomerase activity.<sup>101,114</sup> Together, these data support the proof-of-principle that MSCs could play a relevant role and become an instrumental tool in studies about the etiology and pathogenesis of sarcomas.

It is worth mentioning that in addition to these MSC-based cell transformation models, mouse MSCs frequently undergo spontaneous transformation after extensive in vitro cultured.<sup>99,100,105</sup> Nevertheless, there are conflicting reports about the spontaneous transformation of hMSCs. Thus, it has been reported the spontaneous transformation of BM-derived hMSCs after long-term in vitro culture.<sup>28,101</sup> In contrast, other authors have reported a lack of MSC transformation after extensive in vitro culture<sup>27,115-117</sup> although the occurrence of aneuploidy was observed in some cases.<sup>117</sup> This is a crucial issue as extensive ex vivo expansion of hMSCs is a prerequisite for using these cells in cell-based therapies. Therefore, further investigation efforts are still required to ascertain whether or not MSC transformation is truly common or is an indirect effect of underlying confounding mechanisms<sup>118</sup> or specific pathological conditions. The optimization of culture conditions and the introduction of mechanisms to control the cells after the infusion into patients, such as the introduction of inducible suicide genes in the cells<sup>119</sup> could help to increase the safety of hMSCs based-therapies.

The role of MSCs in tumorigenesis could also be an indirect phenomenon.<sup>120</sup> MSCs are frequently recruited to the site of tissue injury and sometimes, in the appropriate and permissive environment and under stress conditions, this could also represent a potential source of malignancy. Thus, it was described that BM-derived MSCs recruited after infection with *Helicobacter pylori* could be the origin of gastric cancers.<sup>121</sup> Likewise, tumor cells induce the migration of MSCs to the tumor site by the secretion of specific factors.<sup>63</sup> The presence of MSCs in the tumor stroma could facilitate breast cancer metastasis by the secretion of the chemokine CCL5<sup>122</sup> and CCL2.<sup>123</sup> Likewise, the secretion of CCL5 promotes the pulmonary metastasis of osteosarcoma.<sup>66</sup> Recent studies reported that MSCs protect breast cancer cells through the TGF- $\beta$ 1-mediated increase of regulatory T cells.<sup>124</sup> Moreover, BM-MSCs can be the source of carcinoma-associated fibroblast which are supposed to contribute to tumor growth.<sup>125</sup> Furthermore, MSCs seem to create in many cases an optimal microenvironment for the development of several tumors such as multiple myeloma<sup>126,127</sup> or acute lymphoblastic leukemia<sup>128</sup> and BM-MSCs carrying the fusion gene MLL-AF4 were detected in patients of MLL-AF4+ B-acute lymphoblastic leukemia suggesting that MSCs may be in part tumor-related.<sup>129</sup> Activation of anti-apoptotic pathways seems to play a central role in this regard since BM-derived MSC are resistant to chemotherapy-induced apoptosis<sup>103,130</sup> and also contribute to generate drug resistance in tumor cells.<sup>128,131</sup> Several studies have evidenced that hMSCs are highly resistant to ionizing radiation as well as to many chemotherapeutic drugs<sup>102</sup> and this fact could also have important implications for cancer therapy as this chemo and radioresistance could lead to the accumulation of mutations, resulting in MSC transformation and potentially giving rise to secondary tumors.<sup>102,132</sup>

Despite the susceptibility of MSCs to transform or contribute to tumor growth, there are also studies claiming their potential to inhibit tumor growth. Thus, hMSCs can home to tumor sites and inhibit the growth of neoplastic cells as has been shown in models of



gliomas,<sup>133</sup> Kaposi's sarcomas,<sup>134</sup> hepatoma<sup>135,136</sup> and other tumor models.<sup>137</sup> The suppression of Kaposi's sarcoma growth is associated with the inhibition of Akt signaling.<sup>134</sup> Other studies, however, have not found any effect on tumor growth after injection of MSCs.<sup>137</sup>

In summary, all these studies reveal the complex and dual relationship between MSCs and cancer. First, many studies support the potential of MSCs as a promising anti-cancer therapeutic option, either by inhibiting the growth of cancer cells or by serving as vehicles to deliver anti-cancer agents. On the other hand, in line with the well-established link between development, stem cells and cancer, MSCs are being exploited as a target cell for the origin of mesenchymal tumors, especially sarcomas. In addition, whether MSCs and stroma cells form part of the tumoral clone and their potential contribution to the tumor microenvironment is under active investigation. Nevertheless, very little is still known about the mechanisms of MSC transformation and further studies are required to ensure the quality and biosafety of MSCs in present and future clinical trials.

## CONCLUSION

MSCs exist in BM and other tissues and are one of the most promising adult stem cell types due to their availability and the relatively simple requirements for their *in vitro* expansion. These cells contribute to the homeostasis of mesenchymal tissues as well as support for the growth and differentiation of HSCs. A striking feature of the MSCs is their low inherent immunogenicity. Instead, MSCs appear to be immunosuppressive *in vitro*. Their multilineage differentiation potential coupled to their immuno-privileged properties is being exploited for both autologous and allogeneic cell therapies, tissue engineering and regenerative medicine. Additionally, the rapid evolution of experimental data has acknowledged the critical relevance of MSCs as the target cell for either spontaneous or induced cell transformation representing a potential platform for modeling nonhematological mesenchymal cancers such as sarcomas. In this chapter, we have briefly outlined the current status of the MSC field, focusing on their biological characteristics and potential for clinical applications.

Stem cell therapy is rapidly developing and has generated excitement and promise as well as at times some contradictory results. Despite the great promise held by MSCs and although several clinical trials are underway for a variety of malignant disorders, HSCT or regeneration of damaged tissues, it must be recognized that MSCs are poorly defined by a combination of physical, phenotypical and functional properties. As MSCs could be derived from different tissues as starting material, using diverse isolation protocols, cultured and expanded in different media and conditions, the MSC preparations from different laboratories are highly heterogeneous. All of these variables might have implications on: (1) the selection of cell types and the composition of heterogeneous subpopulations; (2) they can selectively favor expansion of different cell populations with totally different potentials and; (3) they might alter the long-term fate of adult stem cells upon *in vitro* culture. The recent controversy about the multilineage differentiation potentials of some specific MSC preparations might be attributed to this lack of common standards. In addition, the most appropriate source of MSCs, method of isolation and expansion, dose to be infused, timing and route of delivery, and adverse biosafety effects-like the potential of MSCs to undergo spontaneous transformation still need to be determined. More precise genetic stability studies, molecular and cellular markers to define subsets of MSCs and the standardization of the protocols for expansion of MSC are



urgently needed. Also, further insights about the immunological mechanisms underlying the immunosuppressive effects of MSCs are still in high demand.

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## NEURAL STEM CELLS AND TRANSPLANTATION STUDIES IN PARKINSON'S DISEASE

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**Abstract:** Parkinson's disease (PD), one of the most frequent neurodegenerative disorders, is primarily caused by the selective degeneration of specific neuronal populations, particularly dopaminergic neurons within the substantia nigra projecting to the striatum (nigrostriatal neurons). The current pharmacological treatments are efficient in the early stage of the disease but with the continuous use of the pro-dopaminergic medication may become less effective and cause motor complications. Cell therapy is an emergent alternative therapeutic strategy to PD and consists in the replacement of damaged neurons by new cells that could help to restore the nigrostriatal pathway. For this purpose, stem cells constitute a promising tool that could provide new sources of cells to be used for experimental transplantation studies in PD, as well as in other neurological disorders. Numerous studies are being made with the use of tissue specific neural progenitors obtained from either fetal or adult nervous systems. In this chapter we will summarize the numerous preclinical and clinical studies performed so far in animal models of the disease as well as in patients and how the use of neural stem cells might improve the current cell therapies.

### INTRODUCTION

Parkinson's disease (PD) constitutes, after Alzheimer's, the most frequent neurodegenerative disorder. It affects over 1 million Europeans and several million people worldwide. PD is primarily caused by the selective degeneration of specific neuronal populations, particularly dopaminergic (DA) neurons within the substantia nigra (SN) projecting to the striatum (nigrostriatal neurons). As these neurons participate in the extrapyramidal motor control, the most prominent PD symptoms are tremor, rigidity,



slowness of movement, and postural instability. Cognitive and behavioral problems, including dementia, are common in the advanced stages of the disease, suggesting the progressive affection of other brain regions. At the neurochemical level, the most noticeable sign of PD is the lack of striatal dopamine resulting from the death of dopaminergic nigrostriatal axon terminals. The current pharmacological treatment of PD is based on the ingestion of levodopa (a dopamine precursor) complemented with the administration of dopamine agonists and inhibitors of dopamine degrading enzymes. These treatments are effective in reversing the motor symptoms in the early stages of the disease. However as the syndrome advances, the continuous use of the pro-dopaminergic medication, that becomes less effective, can lead to motor complications called dyskinesias.

A promising, and intensely investigated, alternative therapeutic strategy to PD consists in the replacement of damaged neurons by new cells that could help to restore the nigrostriatal pathway. The initial goal of this cell-based antiparkinsonian therapy was to restore striatal dopaminergic levels through the grafting of exogenous dopaminergic neurons (cell replacement procedures). Among the various transplantation protocols assayed in the numerous preclinical and clinical studies performed so far, the intraputamenal grafting of fetal mesencephalic dopaminergic neurons has provided the best clinical results. However, the actual clinical efficacy of this procedure has been put into question in two double blind clinical trials, in which it was also reported the appearance of disabling dyskinesias in some cases.<sup>1,2</sup> Moreover, the scarcity of fetal tissue makes this technique difficult to apply to a large number of patients. More recently, the interest of antiparkinsonian cell therapy has shifted to the use of cells that after being grafted into the striatum would deliver trophic factors with protective action on the nigrostriatal neurons affected by the ongoing neurodegenerative process (neuroprotection). Neuroprotective cell therapy pursues the slowing down of PD progression or even the partial reversion of the syndrome.<sup>3,4</sup> The availability of dopaminergic and/or trophic factor producing cells with the appropriate characteristics is a major hurdle in the development of effective novel cell therapies in PD. Therefore, stem cell technologies have recently arisen as highly promising tools that could provide new sources of abundant cells that could be used for experimental transplantation studies in PD, as well as in other neurological disorders.

Stem cells are pluripotent and can spontaneously differentiate, or be induced to differentiate, into a variety of different mature cell phenotypes. Therefore they offer an opportunity to generate large numbers of standardized DA neurons for transplantation.<sup>5</sup> There are two types of stem cells that have been investigated as sources of DA neurons: (1) pluripotent stem cells, which are either derived from the inner cell mass of the embryonic blastocyst (Embryonic Stem cells; ES cells) or obtained by forced reprogramming of somatic cells (induced Pluripotent Stem cells; iPS cells); (2) neural stem cells (NSCs) or precursors, which are endogenous multipotent cells that can be found in the developing or adult nervous system. The use of pluripotent stem cells to treat PD is hindered by poor cellular survival and high risk of teratoma formation.<sup>6,7</sup> For these reasons, most efforts and advances are being made on the use of tissue specific neural progenitors obtained from either fetal or adult nervous systems.

In this chapter we will summarize the basic concepts and general properties applicable to tissue specific NSCs to better understand their suitability for transplantation against PD. The adult NSC niche will be presented as a critical source of signalling, which could help optimize the use of NSCs for medical cell therapy. Finally, we will discuss the attempts that have been made with differentiated dopaminergic cells from various sources to treat PD patients, and how NSCs might be used to improve current cell therapy procedures.



## TISSUE SPECIFIC ADULT NEURAL STEM CELLS

### Concepts and General Properties

For over a century a widely held central dogma in neurobiology has been the belief that neurons in the adult mammalian brain cannot regenerate. However, a number of studies performed in the last decades have shown the existence of progenitor, or neural stem cells, giving rise to new neurons in specific areas of the adult nervous system.<sup>8-10</sup> Adult neural stem cells (aNSCs) retain most of the properties that characterize neural progenitors typically isolated from the fetal nervous system.<sup>11</sup> They are able to proliferate *in vivo* and differentiate into cell types characteristic of neural tissue; mainly glia and neurons.<sup>12</sup> Indeed, self-renewing multipotent aNSCs are considered to be responsible for much of the structural and functional plasticity observed in the adult nervous system.<sup>13,14</sup>

In addition to their role in the normal physiology of the brain, aNSCs have also created wide expectations due to their potential use for tissue repair upon transplantation. These cells are able to proliferate *in vitro* through multiple passages without losing their multipotency and basic properties.<sup>15</sup> They can also differentiate into neurons both *in vitro* and *in vivo* upon transplantation into injured or diseased brain areas.<sup>5</sup> The capacity to control the behaviour of aNSCs and to take advantage of their properties for tissue repair and regeneration crucially depends on knowledge of the molecular signalling involved in the regulation of self-renewal and differentiation. These molecular pathways depend not only on intracellular signals but also on extracellular cues being released by surrounding cells within the niche where aNSCs reside.

Regarding the autonomous intracellular signalling involved in the regulation of aNSC behaviour, numerous studies over the past few years have dramatically expanded our understanding of how self-renewal is regulated at the genetic level.<sup>16</sup> The co-ordinated activities of multiple pathways are required for a stem cell to self-renew, especially because this process involves both proliferation and the maintenance of an undifferentiated state. Some pathways that are necessary for self-renewal appear to regulate only proliferation, while other pathways regulate differentiation potential, or both. Hence, in addition to the niche signalling, aNSCs have intrinsic pathways regulating their function that can converge with the extracellular signals. Some of the best-studied factors regulating neural stem cell self-renewal are those in the notch, Wnt, and hedgehog (Shh) pathways.<sup>16</sup> Understanding the molecular details of these intracellular signals will greatly improve our capability to use aNSCs for therapeutic purposes.

### Stem Cell Niches in the Adult Nervous System

The behaviour of aNSCs is mainly determined by signals released within the niche where they reside.<sup>17</sup> The different cellular elements present in aNSC niches contribute to the formation of a neurogenic environment. Among these elements, the vasculature is attracting special attention, since it has been described that aNSCs are in close contact with the endothelial cells of blood vessels constituting the so called “neurovascular niche”.<sup>18-21</sup> Moreover, numerous factors related with the vascular system are able to promote neural stem cell proliferation and neurogenesis.<sup>22</sup> Hence, endothelium-derived factors will very likely improve our ability to control aNSC behaviour both *in vivo* and *in vitro*.

Neurogenic niches have been described only in a few locations of the adult nervous system. In the central nervous system (CNS), NSCs reside close to the ventricle wall, in an area called the subventricular zone (SVZ). Similar subtype of NSCs has also been described in the subgranular zone (SGZ) within the dentate gyrus of the hippocampus. There is also compelling evidence about constitutive neurogenesis occurring in the adult mammalian olfactory neuroepithelium.<sup>23</sup> Finally, aNSCs giving rise to physiological neurogenesis have recently been described in the adult peripheral nervous system (PNS). These cells are located in the carotid body, a small neural crest-derived, chemosensory organ.<sup>24</sup> Multipotent neural crest-derived stem cells have also been isolated from other PNS structures like the gut<sup>25</sup> or the heart region,<sup>26</sup> however neurogenesis has not been described to occur in these other regions of the adult PNS. Most of the current knowledge on the molecular physiology of the neurogenic niches comes from studies performed in the SVZ and SGZ. Therefore, in the next sections we present a brief description of the major features of these two main CNS stem cell niches.

The SVZ, the largest germinal zone of the adult mammalian brain, lies along the lateral walls of the lateral ventricles.<sup>27</sup> Neuroblasts continuously generated in the SVZ migrate rostrally to the olfactory bulb (OB) where they differentiate into local interneurons.<sup>28</sup> The SVZ neural niche contains, in addition to the ependymal cells lining the ventricle, special astrocyte-like cells that are able to self-renew and give rise to transit-amplifying progenitors, which in turn differentiate into migrating neuroblasts.<sup>29</sup> These primitive astrocytes are quiescent aNSCs residing within the SVZ that interact with ependymal and vascular cells as main constituents of the neurogenic niche. Hence, the adult SVZ constitutes an important reservoir of neural stem cells that can be grown in culture and differentiated into neurons for therapeutic purposes. Regarding PD, however, there is controversy about the real capacity of these SVZ neural progenitors for differentiation into functional DA neurons.<sup>30</sup>

The second well established neurogenic niche in the brain is situated in the hippocampus. The hippocampal formation, located in the temporal lobe of the brain, forms part of the limbic system and seems to play an important role in learning and memory. The hippocampus consists of pyramidal neurons forming the CA regions, and an interior and curved structure called the dentate gyrus (DG), formed by a layer of granule cell neurons. The subgranular layer (SGL) of the DG contains a subpopulation of specialized astrocytes able to give rise to migrating neuroblasts, which in turn differentiate into granular neurons.<sup>31</sup> Hippocampal neurogenesis was originally demonstrated by the immunohistochemical detection of bromodeoxyuridine (BrdU), a synthetic thymidine analogue incorporated into DNA by S-phase cells. Proliferating BrdU positive cells and their neuronal derivatives have been observed in the DG of every mammal examined, including humans.<sup>9</sup> The SGL progenitor astrocytes have radial processes spanning through the granule cell layer, and short tangential processes extending along the border of the granular layer. Understanding what makes the SGL special in supporting the proliferation and neuronal differentiation of multipotent neural progenitors is crucial to be able to control the process and use it with therapeutic purposes.

Several authors have suggested that activation of the SVZ and SGL niches in special situations could induce mobilization of precursors to injured brain areas (i.e., undergoing ischemia or neurodegeneration) where stem cells could differentiate to replace the destroyed cells. However, this possibility is speculative at present and based solely on preliminary experimental observations.<sup>32,33</sup>

## CELL-BASED THERAPEUTIC APPROACHES TO PARKINSON'S DISEASE

For the last decades, there have been numerous preclinical and clinical studies attempting to treat PD in animal models of the disease as well as in patients, using a variety of dopamine-producing cells. Here, we will briefly review the animal models and dopaminergic cells most frequently used in these studies. We will also discuss the potential applicability of stem cells to PD therapy.

### Animal Models of Parkinson's Disease

Development of research on PD depends on the existence of animal models of this disorder, where the effectiveness, advantages and limitations of various therapeutic approaches can be tested experimentally. One commonly used PD animal model is the hemiparkinsonian rat, generated by unilateral stereotaxic injections of 6-hydroxydopamine (6-OHDA) into the SN. The drug is metabolically converted to dopamine and hydrogen peroxide ( $H_2O_2$ ), which subsequently destroys dopaminergic nigrostriatal neurons via oxidative stress. The unilateral loss of DA neurons produces a spontaneous rotational behaviour towards the side of the lesion that can be easily monitored. This model has, however, numerous limitations since neuronal death is not progressive and it is difficult to obtain partially lesioned animals appropriate for the testing of neuroprotective therapies.<sup>34</sup>

Systemic administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a potent mitochondrial complex I inhibitor, in mice produces selective bilateral destruction of DA neurons.<sup>35</sup> The Parkinsonian syndrome in mice is less amenable for behavioural analysis than the syndrome in rats; however, mice are sometimes chosen because MPTP administration requires no surgery and when unilateral intrastriatal transplantation is performed the contralateral side can be used as an internal control. MPTP also produces selective nigrostriatal neuron loss in primates resulting in a parkinsonian model that resembles very much the symptoms presented in humans. MPTP monkeys are often preferred in clinically-oriented studies due to the close similarity of their motor control system to that in man.

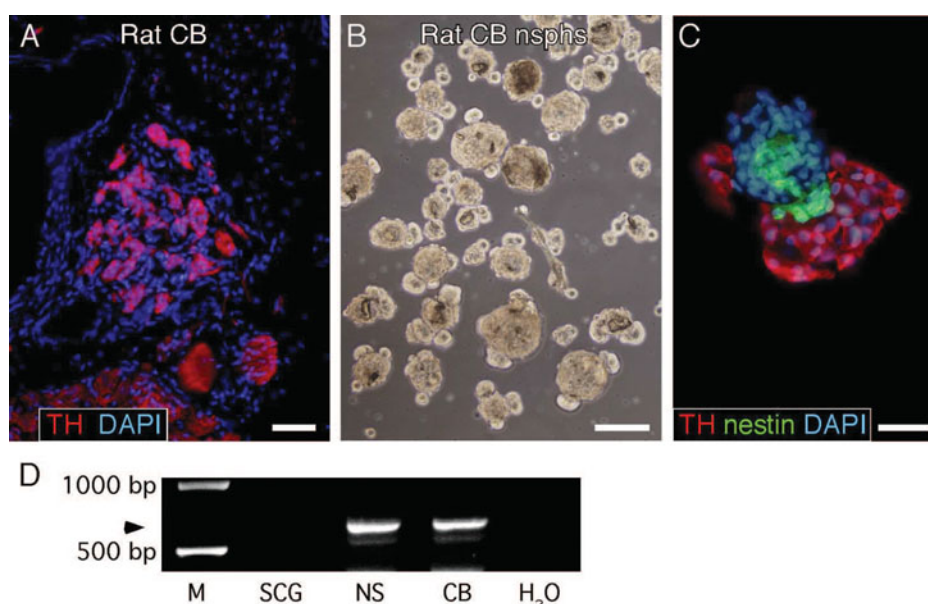
The animal models of PD described above do not recapitulate the progressive neuronal death seen in humans, probably because the cytotoxic agents are administered at high doses and during short time periods. For this reason, and taking into account that some forms of human parkinsonism are familial as a consequence of identified genetic defects, several models of the disease are being developed in genetically modified animals. Among these parkinsonian models, the ones that better recapitulate the human syndrome are the mutated mice for GDNF and LRRK2 kinase.<sup>36,37</sup> Conditional ablation of GDNF production in the adult mouse brain induces a clear and selective degeneration of catecholaminergic neurons,<sup>37</sup> supporting the on-going GDNF-based trials against PD.<sup>38</sup> The LRRK2 transgenic mouse model exhibits cardinal features of PD: Age-dependent and levodopa-responsive slowness of movement associated with diminished dopamine release in the striatum, thus providing a different and valid genetic model for PD.<sup>36</sup>

### Preclinical Studies with Differentiated Dopaminergic Cells

The first cell therapy studies in animal models of PD were performed in the late 1970s in rats using fetal rat dopamine-containing neurons as donors with the aim of restoring striatal dopamine levels.<sup>39,40</sup> After this pioneer work, numerous studies have

been performed in the last 30 years with the objective of establishing the effectiveness of different types of dopaminergic donor cells to recover the parkinsonian syndrome. After the initial transplantation of mesencephalic tissue from rat embryo to rats, further steps included transplantation of mesencephalic dopaminergic neurons, taken from mouse embryos or human fetuses, into dopaminergically denervated striatum of recipient rats or MPTP-monkeys.<sup>41</sup> Besides mesencephalic fetal dopaminergic neurons from different species, other cell types (i.e., adrenal chromaffin cells, and sympathetic neurons from the superior cervical ganglion) have been also tested although with poor results.<sup>42-44</sup>

Among the most promising new experimental approaches developed within the last few years is the intrastriatal transplantation of carotid body (CB) tissue. Autotransplantation of dopaminergic CB cell aggregates has been reported to effect notable histological and functional recovery in parkinsonian rats<sup>45,46</sup> and MPTP-treated monkeys.<sup>47</sup> The CB is a tissue particularly attractive for antiparkinsonian cell therapy because it combines properties necessary for both dopamine cell replacement and neuroprotection.<sup>48</sup> The CB contains highly dopaminergic neuron-like glomus cells that express tyrosine hydroxylase (TH), the rate-limiting enzyme in the synthesis of dopamine (Fig. 1A). Moreover, PCR (Fig. 1D) and transgenic animal studies have shown that these cells produce high levels of the neurotrophic factor GDNF.<sup>48</sup> Hence, besides the transplantation of dopamine-producing cells, other experimental strategies, such as



**Figure 1.** Rodent CB stem cells differentiate in vitro into mature glomus cells. A) Thin section of a rat carotid bifurcation at the level of carotid body and immunostained with antibodies against TH (red) to see the typical clusters of dopaminergic glomus cells within the parenchyma. Nuclei are counterstained with DAPI (blue). B) Bright field picture of typical rat CB neurospheres obtained upon in vitro plating of dissociated cells from the organ. C) Example of a rat neurosphere thin section immunostained to show expression of nestin (green) by progenitors within the neurosphere core and TH (red) by glomus cells within the differentiating blebs. D) RT-PCR showing expression of GDNF (arrowhead) in neurospheres (NS), in whole carotid body (CB) and not in the superior cervical ganglion (SCG). M: DNA marker. Scale bars: 50  $\mu$ m (A and C) and 100  $\mu$ m (B). Modified from reference 24.

transplantation of GDNF-producing CB glomus cells, have been designed to increase the striatal concentration of trophic factors that could eventually induce regeneration of the dopaminergic nigrostriatal pathway.<sup>4</sup>

### **Clinical Studies with Transplanted Dopaminergic Cells**

Transplantation of dopamine-secreting cells in advanced PD patients was initiated in the mid 1980's. Despite highly variable clinical outcomes of these studies, with excellent results reported in selected patients but only modest effects in most cases, overall benefit experienced by the patients stimulated further research and clinical tests. Clinical trials resulting in higher impact have been those employing adrenal medulla,<sup>49</sup> mesencephalic neurons<sup>50</sup> or carotid body<sup>51</sup> as donor tissues. The transplantation of adrenal tissue was soon abandoned due to relatively high morbidity/mortality associated with dual (abdominal and cranial) surgery and the development of other therapeutic approaches. On the other hand, transplantation of fetal mesencephalic neurons constitutes a technology that has dominated clinical trials in cell therapy applied to PD patients for the last 15-20 years.<sup>52</sup> In most of these trials the procedure was generally well tolerated, although some complications associated with cranial surgery were reported. Nevertheless, the main problems encountered in these clinical studies were related to donor tissue. The dissection and manipulation of fetal ventral mesencephalon is technically difficult and results in a mixture of different neural populations with high incidence of cell death upon transplantation. This high cellular mortality increases the need for still larger number of cells for the transplants and human embryos for cell preparation.<sup>53</sup> The problems with donor tissue lead to relatively poor clinical effects worsened by the appearance of severe motor complications due to aberrant striatal synaptic connections between transplanted cells and striatal neurons. Limitations and complications derived from use of fetal mesencephalic tissue have led to the conclusion that such treatment in its current state is not an advisable therapeutic option for PD. Moreover, double-blind placebo-controlled studies on fetal mesencephalic transplantation<sup>1,2</sup> suggest that dopamine cell replacement in the striatum is not an ideal approach for compensating progressive nigrostriatal neuronal loss. A prevalent current belief is that other cell types and methodologies capable of locally delivering the proper cocktail of trophic factors might be more effective in both protecting nigrostriatal neurons from any PD-causing insult and arresting or reversing the neurodegenerative process.

The CB is an organ composed of highly dopaminergic glomus cells (Fig. 1A) whose efficacy for antiparkinsonian cell therapy has been tested in animal models of PD, as described above. A major advantage of CB with respect to fetal mesencephalic neurons is that the former can be used for autotransplantation since its unilateral surgical resection has no significant side effects,<sup>51</sup> thus circumventing most limitations of fetal transplants. Favourable results in preclinical studies encouraged the realization of two pilot Phase I/II open trials to test the feasibility, safety and clinical efficacy of CB autotransplantation in PD.<sup>51,54</sup> In general, these pilot studies have indicated that CB autotransplantation is a feasible and safe procedure with potential clinical applicability to treat PD patients. In addition, CB grafting has demonstrated symptomatic efficacy similar to placebo-controlled mesencephalic grafts. Moreover, these studies suggest that the clinical improvement observed after CB transplantation derive from a true neuroprotective effect of transplanted glomus cells on the nigrostriatal pathway. Finally, although auto-transplantation is conceptually attractive, the amount of tissue



obtained from a single CB appears to be smaller than that needed to consistently obtain significant clinical benefit. As it happened with mesencephalic tissue, the amount of CB tissue available for transplantation is one of the most crucial factors limiting the efficacy of CB cell therapy in PD.

### **Neural Progenitors and Advanced Therapies in Parkinson's Disease**

As mentioned in the previous section, one of the most important problems associated to the use of dopaminergic and/or trophic factor-producing cells for the treatment of PD is the small amount of tissue available for transplantation. This limitation could be overcome by the use of advanced technologies necessary for the *in vitro* expansion of transplantable cells from multipotent neural progenitors. These types of approaches are still in preclinical phase and not yet translated to the clinics, but constitute the most encouraging alternative for PD treatment. The most promising ongoing projects are related with neural progenitors obtained from the fetal ventral midbrain and from the adult CB.

Recently, a method has been reported for generating large numbers of DA neurons based on expansion and differentiation of ventral midbrain fetal stem cells.<sup>30</sup> Mouse ventral midbrain neurospheres have been expanded with FGF2 and differentiated with sonic hedgehog and FGF8 to generate fair amounts of DA neurons with intrinsic electrophysiological properties of midbrain DA cells. Transplantation of these cells into parkinsonian mice resulted in significant cellular and functional recovery. Importantly, no tumors were detected and only a few transplanted grafts contained sporadic nestin-expressing progenitors.<sup>30</sup> It has been estimated that this technique would allow the production of as many DA neurons from one single human embryo as was previously described for 6 embryos, clearly increasing the efficacy of the process. However, dopaminergic differentiation of progenitor-derived fetal midbrain neural stem cells is achieved by transfecting the cells with Wnt5a, a transcription factor involved in dopaminergic specification. The transgenic character of the resulting DA neurons limits the clinical applicability of the technique.

Similar to the case of mesencephalic neurons, CB-based cell therapy is also trying to take advantage of stem cell technology to improve the efficacy of transplantation treatment. A new population of adult CB-specific neural progenitors have recently been described within the organ.<sup>24</sup> These cells are neural crest-derived and able to form neurospheres *in vitro* (Fig. 1B). Moreover, progenitors within these neurospheres spontaneously differentiate into dopaminergic glomus cells, which are histologically and functionally similar to those studied in the organ *in situ* (Fig. 1C and D).<sup>24</sup> After only 10 days *in vitro*, rat cultures of CB progenitors allow the production of 3 times the number of glomus cells normally present in the whole organ, thus making CB expansion possible. We have also shown production of GDNF from glomus cells differentiated in the neurospheres (Fig. 1D), however the efficacy of CB neurospheres on the amelioration of PD symptoms in animal models of the disease is still under evaluation. CB progenitors are also present in adult and elderly human CB parenchyma and they can form neurospheres *in vitro* (data not shown). Therefore, whether human CB stem cells will offer an opportunity for tissue expansion prior to transplantation is a question that must be addressed in future experimental work. In summary, the examples described above indicate that tissue-specific progenitors offer new prospects for the improvement of cell-based therapies against PD.

## CONCLUSION AND PERSPECTIVES

PD patients are currently treated with a variety of pharmacological tools, including L-DOPA, dopaminergic agonists and inhibitors of dopamine degrading enzymes. The current surgical treatment of PD is mainly based on the use of deep brain stimulation. While these treatments provide symptomatic relief, none of them change the course of the disease. There is therefore a clear need for restorative and regenerative approaches, including cell therapy. It is only in the last 25 years that experimental and clinical studies have systematically addressed the possibility of neural reparation by means of cell therapy. The original goal of cell therapy was to restore function by replacement of dead cells with healthy ones. However, in most recent studies the primary interest has shifted from cell replacement to neuroprotection, in the hope that application of the proper cocktail of trophic factors released from cells grafted at injured sites or administered systemically would either prevent neuronal damage or activate intrinsic regenerative mechanisms leading to restoration of destroyed neurons or synaptic connections.

Fetal mesencephalic and carotid body dopaminergic tissues have mainly been used for cell therapy to replace or recover DA neuronal loss during the course of PD. These cells have been grafted in the target of midbrain DA neurons, the striatum, in an attempt to facilitate axonal sprouting and re-innervation. In the last 25 years, numerous PD patients have been grafted with human fetal mesencephalic as well as other, i.e., carotid body, tissues. As expected from a new experimental therapy, results have ranged from failure to success. While success provided proof of principle that cell therapy can work in PD, failures pointed out to very important issues that need to be improved in order to develop a viable and competitive cell therapy for PD. One of the most important issues highlighted by clinical trials has been the small amount of tissue available for transplantation, due to either difficulties in obtaining human fetuses or the small size of the human CB. Stem cells arise as a promising tool to increase biomass of DA cells in vitro suitable for transplantation. Important effort is being made on studying the use of fetal or adult tissue-specific neural stem cells for differentiation in vitro into dopaminergic cells. These studies are currently in preclinical phases but will likely encourage clinicians to initiate new clinical trials with PD patients.

PD cell-based therapies have also been much influenced by the development of ES and iPS cell research, as these cells have the potential to give rise in vitro to any cell-type of the adult organism. Pluripotent cells are generally thought to offer a promising source of neurons suitable for cell replacement therapy in PD and other disorders. Most investigators in the field, however, are aware that translation of pluripotent cells to the clinical setting is confronted with numerous limitations and unsolved problems. Differentiation of pluripotent cells to mature neurons is still not well controlled and so the possibility of tumour generation is high. In addition, short lasting viability of neurons derived from human pluripotent cells compromises their use in cell therapy. Hence, efficient generation of neural subtypes with correct phenotype from pluripotent cells remain a challenge. Consequently, major hurdles still lie ahead in applying human pluripotent cell-derived neural cells clinically. Excellent reviews have recently addressed the potential of pluripotent stem cells in PD and the way these cells are changing the landscape of cell therapy against neurodegenerative disease.<sup>3,55-57</sup>



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# BIOLOGICAL IMPACT OF HUMAN EMBRYONIC STEM CELLS

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**Abstract:** Research on human embryonic stem cells (hESCs) and induced pluripotent (iPS) stem cells is currently a field of great potential in biomedicine. These cells represent a highly valuable tool for developmental biology studies, disease models, and drug screening and toxicity. The ultimate goal of hESCs and iPS cell research is the treatment of diseases or disorders for which there is currently no treatment or existing therapies are only partially effective. Despite the disproportionate short-term hopes generated, which are putting too much pressure on scientists, the international scientific community is making rapid progress in understanding hESCs and iPS cells. Nonetheless, great efforts have to be made to provide an answer to still quite basic questions concerning their biology. Moreover, translation to clinical applications in cell replacement therapy requires prior solution to ethical barriers. The recent development of iPS cells has provided a strong alternative to overcome ethical issues concerning hESCs. However, an in-depth characterization of their genetic and epigenetic features, as well as their differentiation potential still remains to be undertaken. This chapter will describe, precisely, what the critical issues are, where scientific and ethical barriers stand, and how we are to overcome them. Only then, we shall finally discover whether hESCs and iPS cells will allow building reproducible disease models, and whether they really are a safe tool, with great potential for regenerative medicine.

## INTRODUCTION

Research on human embryonic stem cells (hESCs) and induced pluripotent (iPS) stem cells is currently a field of great potential in biomedicine. These cells represent a

highly valuable tool for the design of models to study human embryonic development and diseases which have their roots in the embryonic phase, as well as for the screening of new drugs and as a source of cells to develop therapeutic strategies in regenerative medicine. The ultimate goal of hESC and iPS research is the treatment of diseases or disorders for which there is currently no treatment or existing therapies are only partially effective.<sup>1</sup>

The first ESC line was established in 1981 by Sir Martin Evans, using mouse embryonic stem cells (mESC).<sup>2</sup> The use of these cells allowed specific genetic modifications to be made in mice. Sir Martin Evans was awarded the 2007 Physiology and Medicine Nobel Prize for his contribution to the development of animal models based on genetic manipulation of mESC for the study of diseases. In 1998, the group led by Prof. James Thomson<sup>3</sup> in the USA succeeded in deriving the first hESC line from a blastocyst in the pre-implantation phase. This scientific milestone launched a new field in biomedical and regenerative medicine research, full of expectations.

The general public very quickly became aware of the potential impact of hESC research, creating disproportionate short-term hopes due to a lack of objective information on the issue. The coverage in the mass-media and the placing of hESC research at the centre of scientific policy (which is evidently political attractive and has occurred in many countries) has led to a situation where the natural, exclusively scientific rhythm that hESC researchers need to keep to in order to proceed with their work cannot meet the expectations of the media and the public. This is creating confusion and generating pressure on scientific production which could seriously affect the quality of the research carried out. Indeed, this situation might come to jeopardize the minimum necessary standards of the scientific work which should, in the future, provide an answer to the question of whether hESCs/iPSCs are the source of cells with greatest potential for the development of efficient and safe cell therapies.

Furthermore, the more recent development of iPS cell lines provides a new source of cells capable of self-renewal and differentiation into all types of somatic cells. Cellular reprogramming has thus become the flavour of the month. Pluripotency can be induced in human somatic cells by ectopic expression of few transcription factors.<sup>4</sup> The ability to reprogram somatic cells will allow the generation of an unlimited platform of cells pushed back in development, molecularly and functionally indistinguishable from hESCs which may represent a useful potential tool to facilitate the development of a reliable method to prospectively generate in a reproducible manner lineage-specific derivatives and would become an ideal cellular platform to generate patient-specific stem cells in the near future. However, an in-depth characterization of the differentiation potential of iPS cells still remains to be undertaken.

This chapter will describe, precisely, what the critical issues are, where scientific and ethical barriers stand and how we are to overcome them in order to meet some of the expectations raised by hESCs and iPS cells and, as part of this, build reproducible cellular models of disease.

## **HUMAN EMBRYONIC STEM CELLS BIOLOGY**

The unique nature of hESCs is defined by two properties: Self-renewal, thanks to which they are able to perform an indeterminate number of symmetrical cell divisions to maintain their population, and pluripotency, which allows them to differentiate towards

any cell-type representing the three germ layers: Ectoderm, mesoderm and endoderm (see Fig. 1).<sup>5</sup>

The biomedical potential of hESCs is granted by their pluripotency, but this property is a double-edged sword: The very plasticity which allows to turn them into hundreds of different cell types also makes them harder to control and their genetic stability more difficult to maintain; the hardest challenge of all being the optimization of cell differentiation protocols, that will guarantee success in obtaining mature and functional cell phenotypes for use in cell replacement therapies.<sup>6</sup> Besides, the population of clinical interest must be absolutely homogeneous, at the right level of differentiation and free from contaminating undifferentiated cells.<sup>6</sup>

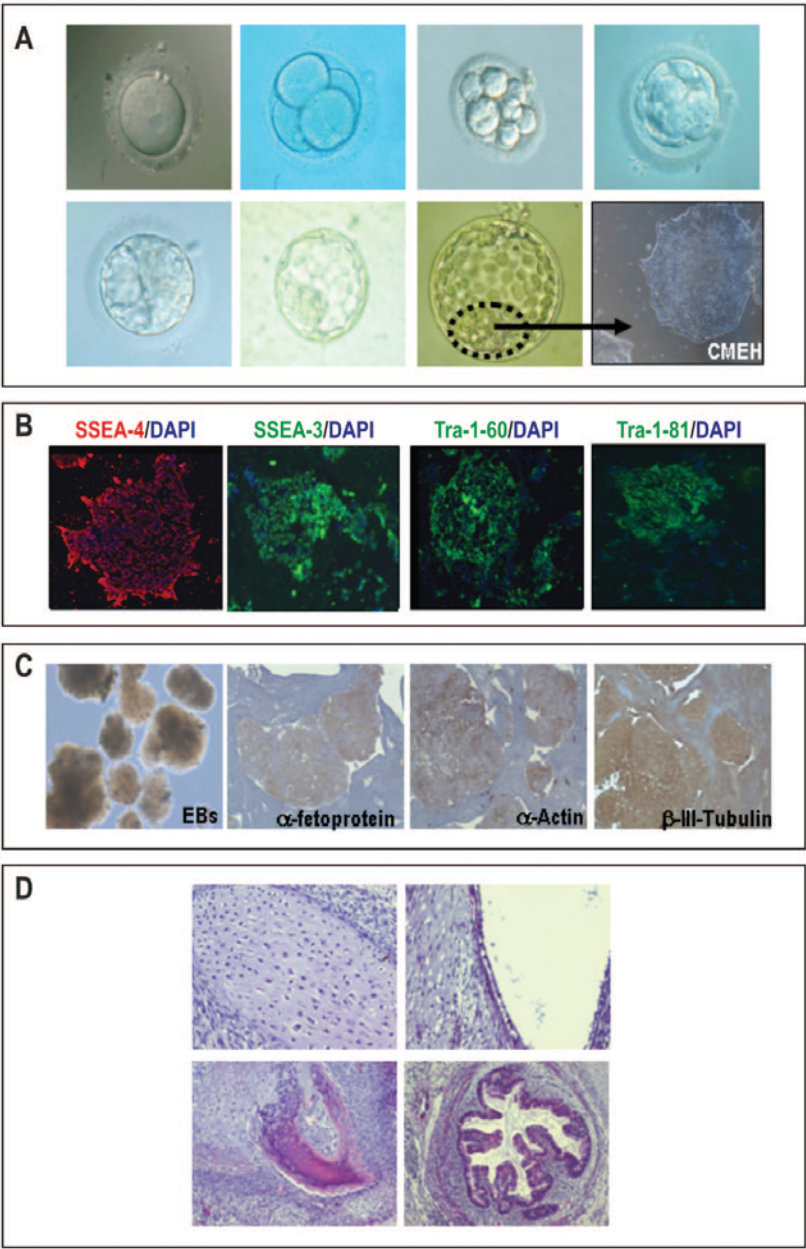
### Human Embryonic Stem Cells Derivation

hESCs are derived from the inner cell mass (ICM) of blastocysts donated by couples undergoing in vitro fertilization (IVF) cycles.<sup>7,8</sup> In most cases, the ICM is isolated from trophoblast cells using either immunosurgery or mechanical dissection.<sup>9</sup> However, there is a relatively new and successful technique employing a laser dissector (see Fig. 1). This method involves accurate destruction of trophoblast cells with laser pulses to obtain a pure culture of ICM cells.<sup>10</sup> Recently, hESC lines have been derived from a single cell (blastomere), taken from an embryo at the 8- to 10-cell stage, following the same method used for pre-implantation genetic diagnosis.<sup>11</sup> Although this method seems to guarantee the viability of the embryo, further in depth studies are needed in order to rule out possible long-term side effects. Of interest, we have recently reported that mesenchymal stem cells facilitate the derivation of hESC cells from cryopreserved poor-quality embryos and that the ROCK inhibitor Y-27632 increases post-thaw embryo survival.<sup>12,13</sup>

### Culture, Characterization and Genetic Stability

Initially, for ICM-derived hESC growth, mouse embryonic fibroblasts (MEF) were used as feeder and support cells.<sup>3</sup> Methods developed later employed cells from various human tissues as feeder cells, thus avoiding the use of cells of animal origin that would limit future applications of cell therapy.<sup>14</sup> In recent years, efforts have been focused on the optimization of culture systems which avoid the presence both of animal origin products and of feeder cells, so that risks are minimized when it comes to pathogen contamination of the cells, considering their future use in cell replacement therapies.<sup>15</sup> Using feeder cell conditioned media and chemically defined media, which contain a variety of combinations of growth factors as substitutes for traditional sera, and growing cultures on extracellular matrices, such as Matrigel® or on matrices produced from human origin extracellular matrix proteins, have made it possible to maintain undifferentiated hESC cultures for a large number of cycles without observing any genetic alterations.<sup>10-17</sup>

Cultures of hESC lines should be subjected to routine monitoring of their cell characteristics and their genetic stability. hESC characterization must be carried out following the criteria established by the International Stem Cell Initiative, according to which hESC must show a similar expression of certain markers, characteristic of their qualities of self-renewal and pluripotency. As surface markers, they must express the antigens: SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, GCMT2 and GCT343. In addition, they must express a set of genes associated with the maintenance of pluripotency: Nanog, POU5F1 (OCT4) and 14 other genes the expression of which is associated



**Figure 1.** Derivation and characterization of hESCs. A) Representative images of human embryonic development from the zygote to the hESC, going from 2 cell-stage through the morula and, eventually the blastocyst. B) Phenotypic characterization of hESC, by immunocytochemical expression of antigens SSEA-3, SSEA-4, Tra-1-60 and Tra-1-81. C) hESC ability to differentiate in vitro by forming embryoid bodies which differentiate into tissues of the three germ layers (alpha-fetoprotein [endoderm], actin [mesoderm] y  $\beta$ III-tubulin [ectoderm]). D) Representative images of teratomas generated upon injection of hESCs into immunodeficient mice. The bottom right panel shows a normal testis transplanted with PBS revealing normal seminiferous tubules.



with Nanog. Finally, hESCs must be capable of creating teratomas when injected into immunodeficient mice thereby proving their ability to differentiate into the three germ lines *in vivo*.

One of the basic requirements hESCs and iPS cells must meet for their future use in regenerative medicine is genetic stability. Amongst the scientific community, top priority is being given to the investigation of culture and expansion methods that will guarantee chromosome integrity and genetic stability. Therefore, not only are conventional studies on karyotype monitoring required but also other more accurate techniques, such as FISH (fluorescent *in situ* hybridization), SKY (Spectral Karyotyping), CGH Array (comparative genomic hybridization) and array mapping and single nucleotide polymorphism (SNPs) should be considered. So far, various chromosomal changes have been observed in certain hESC lines. Some of these changes are associated with oncogenesis and cell transformation; however, the molecular causes responsible for this phenomenon remain unknown.<sup>18</sup> Several hypotheses, such as the duration of *in vitro* cultures, the method of culture or the method of dissociation of hESC colonies using enzymes are being considered. Nevertheless, the possibility that the observed chromosomal anomalies might be a mere intrinsic property of each hESCs cannot be ruled out.<sup>18-20</sup>

Besides maintaining current efforts to develop genetic markers that will guarantee hESC stability, it is also important to develop functional markers that will act as quality indicators of undifferentiated cultures and will help with the process of obtaining mature phenotypes when applying lineage-specific cell differentiation methods. One of the less studied aspects relevant to these objectives is the bioenergetic and metabolism of the hESC, particularly the contribution of the mitochondrial genetic and function.

### Cell Differentiation Strategies

The development of the enormous potential of hESC depends upon successful optimization of lineage-specific cell differentiation methods that will guarantee the viability and homogeneous differentiation of individual tissue-specific progenitor cells.

The vast majority of differentiation protocols described so far are based on the formation of embryoid bodies in culture, giving rise to a heterogeneous cell population of the three germ layers: Endoderm, mesoderm and ectoderm. Embryoid bodies are commonly treated with combinations of growth factors and specific morphogens which may boost lineage-specific differentiation cells at different stages of development.<sup>21,22</sup> Another experimental approach is the coculture of hESC with adult stromal cells, in which the latter act as a growth and differentiation niche for the pluripotent stem cells. This is a way of achieving, for instance, hESC differentiation into neuron precursors, hematopoietic precursors or dendritic cells.<sup>23,24</sup>

Currently, cell differentiation strategies are being designed by growing hESCs on extracellular matrices and using various combinations of chemically defined media (CDM), free from animal-derived components and loaded with a variety of combinations of growth factors and morphogens, such as basic fibroblast growth factor (bFGF), Noggin, BMP (bone morphogenic protein) and Activin A, which genetically direct the differentiation process into one particular cell-type and lineage. The aim is to avoid the step involving the formation of embryoid bodies and the use of cell cocultures, which imply generation of heterogeneous cell populations and less chemical control over the factors conditioning the differentiation process, and thereby to obtain homogeneous cell populations, both in lineage and cell-type as well as in maturity and functionality.<sup>25</sup>



Another useful strategy when it comes to hESC differentiation towards a specific cell lineage is based on genetic manipulation through over-expression or inhibition of certain genes, together with the creation of models for diseases of a genetic origin which start at prenatal stage and for which there are still no satisfactory models, as is the case with certain types of leukaemia in children, paediatric neuroblastoma and mitochondrial genetic diseases with a rapid neonatal development.<sup>26</sup>

Before differentiation strategies can be claimed to be successful, and prior to embarking on clinical studies, it is necessary to verify that the cells obtained are functionally active and have a mature and stable phenotype. In order to do this, genotypic and phenotypic analysis must be carried out and functional markers must be developed so that, after completion of the differentiation process, it can be demonstrated that cells have reached a more mature metabolic state, as well as that they meet a set of minimum specific functional parameters for each type of cell lineage.

### **Metabolism and Culture Adaptation of the hESCs: An Uncontrollable Biological Artifact?**

Human ESCs are thought to be an adaptation to cell culture conditions, because the properties of the ICM are transient in nature, whereas cultured hESCs divide indefinitely without losing pluripotency. The study of the energetic and oxidative metabolism of hESCs has barely been studied. The role played by the mitochondrial genetics and function in the hESC biology remains to be elucidated. How mitochondrial adaptation upon derivation itself, or as a response to different culture approaches, may affect the pluripotency of the hESC lines need to be addressed. Only in the past few years several reports have begun to answer some of these open questions, particularly focused on the mitochondrial biogenesis progression through hESCs differentiation.<sup>27,28</sup> It would also be desirable to find out how the maintenance of hESC cultures is affected by possible mitochondrial genome changes, considering that each hESC line has been derived from embryos with different mitochondrial genetic haplotypes or polymorphic changes in their mitochondrial genome. A detailed knowledge of the mitochondrial contribution to hESC biology will allow the identification of new functional markers. Moreover, having accurate data concerning possible differences of mitochondrial biogenesis, metabolism and adaptation among distinct hESC lines would be very useful to define complementary functional criteria. Such criteria could then be used to analyse hESC culture quality as well as to assess the efficiency of the cell differentiation protocols applied.

It must be stressed, with regards to the need for a greater understanding of hESC metabolism and mitochondrial function that hESC lines are derived from blastocysts which are in a hypoxic environment, where the mitochondria/cell ratio and the rate of aerobic metabolism are quite low. However, the presence of mitochondria is crucial, given that several pathways that control the balance between proliferation and cell differentiation depend upon the mitochondrial function.<sup>29</sup> A key issue is that once hESCs have been derived and subjected to continuous culture, the cells experience a sudden environmental change when they are transferred from their *in vivo* hypoxic microenvironment (1-5% O<sub>2</sub>) to normoxic culture conditions (21% O<sub>2</sub>). Actually, the above definition of “normoxic” conditions, which refers to the common cell culture conditions of 21% O<sub>2</sub> could be confusing, since 1-5% O<sub>2</sub> is the *bonafide* normoxic microenvironment within the blastocyst, and therefore, 21% O<sub>2</sub> represents an aberrant hyperoxic environment. The change in the microenvironment of hESC cultures and their subsequent adaptation to their new

aerobic surroundings may provoke a change in hESC metabolism, and accordingly in their mitochondrial function. Whether or not “mitochondrial metabolic reprogramming” impacts molecular mechanisms of cell proliferation and differentiation is a still unresolved question which should be addressed in order to attain a better understanding of the nature of hESC biology. It is also important to assess whether this set of alterations, which have a direct effect on hESC biology itself, actually turn them, in their *ex vivo* environment, into a biological artifact. This might be the key for deciding, in the near future, whether hESCs are, in fact, what they have so far been thought to be: A biological tool of great potential for regenerative medicine.

Some researchers have recently begun to study the advantages of growing hESCs and other types of somatic stem cells in hypoxic conditions (3–5% O<sub>2</sub>). Under these conditions they have found that the self-renewal capacity is higher, culture stability increases and spontaneous differentiation occurs less frequently. Moreover, the efficiency of embryoid bodies formation is also significantly improved.<sup>30</sup> We have also recently begun to maintain hESC cultures under 3% O<sub>2</sub>, finding a more stable long-term culture of undifferentiated colonies with a stronger expression of pluripotency markers (unpublished observations). These preliminary data clearly suggest the need to further address the questions posed in this section.

On the other hand, gaining knowledge about the mitochondrial role in hESCs biology will provide an important potential platform to improve the efficiency of cell reprogramming and iPS cells generation. Considering that the somatic cells target for reprogramming have to travel back in development towards an embryonic-like state in their genetic and epigenetic programs, a “mitochondrial reprogramming” is also expected to take place to attain the adaptation of the mitochondrial genetic and function to the novel embryonic-like state. Therefore introducing experimental factors to better modulation of the mitochondrial change may improve the efficiency of the reprogramming approaches. To date, only a work published this year has focused in this direction, demonstrating that human iPSC lines have similar mitochondrial regulation and stress defence mechanisms to hESC lines.<sup>31</sup>

## IMPACT OF HUMAN ESC: TECHNICAL AND ETHICAL BARRIERS

So far, safe and efficient use of hESCs in cell replacement therapy has been limited by the need to overcome a series of technical and ethical barriers. Ever since this field of biomedical research began, back in 1998 when Thomson and colleagues derived the first hESC line, an unprecedented debate has exploited concerning the possible advantages and disadvantages of using hESCs in cell therapy, compared to the use of adult stem cells (ASCs).

As aforementioned, there are many biological factors underlying the characteristics of hESCs and their adaptation to *ex vivo* cultures which remain unknown but need to be controlled if we are to propose the use of hESCs as potent and safe option in future cell replacement strategies and drug discovery. Today, only some specific ASCs can be considered clinically safe and realistically applicable to regenerative medicine, though it should be remembered that even in their case long-term efficacy has not yet been well-established. In the case of hESCs, it is necessary to solve several technical and ethical problems before being able to properly grant these cell lines the high status that disproportionate expectations have already conferred on them, all too soon. There is a wide range of items to be addressed: From ethical issues concerning the use of embryos

for hESC derivation and its alternative, consisting of somatic nuclear transfer to generate patient-specific hESCs, to purely biological issues related to their genetic control and stability, their correct characterization and culture, epigenetic changes and chromosome abnormalities during their maintenance, as well as the use of efficient cell differentiation strategies that will ensure their safety and efficiency, so that the risks of triggering immune responses and tumour formation are minimised. In this section we will consider these issues and some of the various strategies currently in use to address/overcome them.

### **Human ESC Derivation: The Need to Find Ethically Validated and Potentially Applicable Alternatives**

The main ethical objection in some western countries to hESC derivation lies in the destruction of life in the form of an embryo. A logic solution to this problem would be to derive hESCs without the need to use embryos. There are currently a number of experimental strategies which aim to generate patient-specific pluripotent stem cells.

#### *Nuclear Reprogramming through Cell Fusion*

This method consists in the fusion of already existing hESCs with adult somatic cells, in order to create a cell line which would have ESC properties while maintaining the adult somatic cell genotype.<sup>32</sup> However, once the cell hybrid has been reprogrammed to acquire hESC properties, a very complex technical procedure is then required to eliminate ESC chromosomes from the tetraploid fusion cell. This procedure has not yet been optimized and it is a costly and time consuming alternative, as well as carrying the risk of affecting future genetic stability of the cells.

#### *Somatic Cell Nuclear Transfer*

Somatic nuclear transfer (SCNT) is based on the introduction of the nucleus of a somatic cell into a previously enucleated egg cell with the aim of creating a cloned embryo. hESCs derived from this embryo would be genetically identical to the donor of the somatic nucleus. This technique is also known as therapeutic cloning.

The production of live animals using SCNT has shown that the epigenetic status of somatic cells, even of totally differentiated adult somatic cells, is stable but not irreversible, as it can be reprogrammed to an embryonic state, capable of directing the development of a brand new individual.

This technology provides a very interesting experimental approach to studying key epigenetic mechanisms which control embryonic development, as well as of models of disease whose origin can be traced to genetic alterations that occur in the process of embryonic development. Nevertheless, there is still ethical conflict when it comes to its potential therapeutic use to generate patient-specific hESCs. Besides which, it has certain technical weaknesses, such as the extremely poor efficiency of the cloning process and a lack of molecular-genetic understanding of the process itself.<sup>33</sup>

When using experimental techniques which involve cell fusion or SCNT it must be taken into account that both approaches can produce cell entities with mitochondrial heteroplasmy, meaning by this that by cell fusion and by nuclear cell transfer mitochondria carrying a different genome to that of the recipient cell can be introduced. This situation may alter the future mitochondrial inheritance, due to cohabitation of mitochondria with

different genetic haplotypes or polymorphisms. It can also have a direct effect on the functional viability of the derived hESC.

#### *hESC Derivation from a Single Blastomere*

One or two isolated cells from an embryo at the pre-implantation stage may generate a whole new embryo. The technologies to isolate one single cell from the blastocyst (required, for example, in pre-implantation genetic diagnosis, PGD), and then to generate a cell line from it, are already established. The rest of the embryo may be placed back in the uterus to continue its development.

Recently, Klimanskaya and colleagues have established a hESC line by extracting a single blastomere.<sup>11</sup> Their results offer an experimental alternative which would avoid part of the ethical conflict regarding the possible destruction of life by the use of human embryos to derive hESC lines. However, this technology is still quite complicated to implement and there is no total guarantee of its success and viability. Indeed, so far, there is not a single consistent long-term study providing data on the health of babies born from transferred embryos which have undergone PGD.

#### *Induced Reprogramming of Somatic Cells through Defined Factors*

Genetically activated induction of adult somatic cell dedifferentiation is another option for obtaining ESC-like cells. Takahashi and Yamanaka first succeeded at establishing an unprecedented experimental approach to obtain somatic cells reprogrammed back to an almost embryonic status.<sup>34,35</sup> These authors showed that retroviral expression of four transcription factors (Oct4, Sox2, c-myc and Klf4) in adult fibroblasts and subsequent selection by activation of Oct4 from the Fbx15 gene were sufficient to isolate what have been called induced pluripotent stem (iPS) cells. These iPS cells which had an activated Fbx15 were pluripotent in terms of their enormous ability to create teratomas, but they failed to produce chimeric mice. These cells were dependent on the ongoing viral expression of Oct4 and Sox2, while endogenous expression of Oct4 and Nanog (key transcription factors for maintaining ESC pluripotency) was not detectable. In terms of the safety for future potential therapeutic applications using iPS cells, it must be stressed that c-myc and Klf4 are oncogenes. Therefore, the retroviral induction of their expression in iPS cells may imply a potential risk of oncogenic transformation either by the oncogenes themselves or due to retroviral insertional mutagenesis.

Hence, the initial approach for iPS cells generation was based on the retroviral-mediated expression of oncogenes such as c-myc and Klf4, and on their subsequent selection, using drugs to activate Fbx15, Oct4 or, in a further work Nanog. These two experimental requirements would compromise future potential applications of in vitro cell reprogramming to cell therapy in humans, given that mice derived from iPS cells frequently develop cancer,<sup>35</sup> and that generation of human iPS cannot be carried out through selection of genetically modified donor cells. Some of these experimental limitations have already been overcome in a very short time period. To avoid the risk of tumours in chimeric mice derived from iPS cells, several groups have very recently demonstrated that, although it does make the process more costly and less efficient, c-myc is not absolutely necessary for cell reprogramming.<sup>36-38</sup> Although mice generated from these iPS cells did not develop c-myc-induced tumours,<sup>36,37</sup> it cannot be guaranteed that the expression mediated by

retroviruses for other factors used, such as Oct4, will not induce tumour development in the longer-term. As far as avoiding genetic modification of donor cells in order to generate iPS, totally reprogrammed iPS cells have already been obtained from genetically unmodified mouse and human fibroblasts and many other adult somatic tissues. They were selected exclusively according to morphologic criteria, since the rate of reprogramming was sufficient to enable them to be detected in culture.<sup>38-40</sup>

Another important factor considering the potential future application of the iPS cells is the risk of the viral chromosomal insertions into the genome of the reprogrammed cells. Several techniques have been recently developed to remove the transgenes after reprogramming or to avoid the use of viral-mediated approaches such as the Cre-recombinase excisable-based system that removes the transgenes after iPS cell generation has been successfully employed,<sup>41</sup> the generation of transgene-free iPS cells by the *piggyback* transposon<sup>42,43</sup> and the direct delivery of reprogramming proteins.<sup>44</sup> The most challenging approach would be an efficient method to induce reprogramming by using only soluble factors that may orchestrate the genetic, epigenetic and cellular events necessary for reprogramming adult somatic cells.

### **Definitive Culture Systems Free from Xenobiotic Risk**

As mentioned in the previous section on hESC biology, any kind of animal contamination must be strictly prevented if these cells are to be used in cell replacement therapy. Specifically, feeders and other support cells of animal origin used in the process of derivation or culture as well as animal sera used for the expansion of cultures are sources of pathogenic and xenogenic agents which might provoke immune responses and their presence must be avoided.

Some laboratories are already successfully culturing hESCs on chemically defined culture media, on human feeder cells or even on feeder-free systems. Another very important step that has been taken in this context is the derivation of an hESC line under animal product-free conditions.<sup>21</sup>

### **Control of Post-Transplant Tumour Risk**

One of the most evident risks associated to the use of hESCs in cell therapy would be the development of tumours—teratomas—as a consequence of the presence of a population of contaminating undifferentiated cells. Several studies carried out on hESCs and on differentiated cells derived from hESC proved that after subcutaneous, intramuscular or testicular injection of such cells into adult mice, the animals developed teratomas.<sup>45</sup>

One of the technical problems that need to be solved in order to minimise the risk of developing tumours, in the future use of hESCs in regenerative medicine, is the failure, so far, to obtain efficiencies higher than 99% in the production of a pure differentiated cell population using current direct hESC differentiation approaches. In a cell culture prepared for therapeutic use, even the presence of one single undifferentiated embryo cell could potentially generate a teratoma. A way of guarding against this would be to confirm negative expression of the transcription factors involved in the maintenance of pluripotency, such as Nanog and Oct4. However, given the complexity of the genetic interactions that control cell pluripotency, this would not be a definitive solution. Other more accurate strategies could be based on the generation of hESCs in which it was

possible to perform negative selection in culture, through the use of toxic compounds which would eliminate any undifferentiated cells once the protocol of cell differentiation is over. Yet another alternative would be to separate differentiated cells by fluorescence activated cell sorting (FACS).

### **Genetic Instability: Epigenetic Changes, Chromosome Abnormalities and Adaptation to the Culture Environment**

Animal cloning experiments in mice using ESCs (instead of somatic cells) demonstrated that there was inefficient control over the reprogramming of the genetic material inserted by the embryonic cells, since many of the genes required for early embryonic development were found not to be activated.<sup>46</sup>

Due to variations in epigenetic regulating mechanisms, hESC lines may differ from one to another in their gene expression profile. Such epigenetic changes in hESCs may be divided into: (1) epigenetic changes in pre-implanted embryos used to derive hESC lines; and (2) epigenetic changes stemming from the maintenance of hESCs in culture.

There is great variability regarding the conditions and types of culture used with hESCs, even within the same laboratory. The process of establishing and maintaining cell lines requires cells to adapt to the culture conditions, which are far from the *in vivo* environmental conditions and cell niche. This adaptation may have an effect on genetic stability and may provoke chromosome aberrations. Already several chromosome changes have been described in certain hESC lines, so it can be concluded that they do not constitute a random event, but rather a reproducible phenomenon.<sup>18,45</sup> Regular screenings must therefore be performed by cutting-edge technologies including G-banding, FISH, CGH, SKY, SNPs, etc., in order to avoid using any hESC line presenting genetic instability for potential therapeutic applications.

### **Immunotolerance and Transplant Rejection**

Another challenge that must be faced before attempting therapeutic application of hESC lines is the prevention of rejection once they are transplanted. Although hESCs express low level of major histocompatibility complex class I (MHC class I) molecules and costimulatory molecules, the level increases moderately in hESC-derived cells, which could trigger an immune response in the recipient of the cells. There are several alternative experimental approaches to creating immunotolerance. One of them would be to carry out SCNT using a somatic cell from the future recipient, although this option runs into previously mentioned disadvantages: Ethical issues, very low efficiency, mitochondrial heteroplasmy and presence of histocompatibility antigens in the mitochondria of the egg cell used. Another alternative option would be to generate hematopoietic chimeras to avoid the use of immunosuppressive drugs in transplanted patients. This would imply derivation of hematopoietic cells and of cells specifically required for the transplant from the same hESC line. After inducing immunosuppression in the recipient, both cell derivatives, which would be perfectly compatible, would then be transplanted.<sup>47</sup>

Last but not least, patient-specific iPS cells would be an advantageous approach to avoid rejections after transplantation, if in the future, the development of the methodologies for creating iPS cells are sufficiently optimized to be considered safe for therapeutic applications.



## CONCLUSION

While there are many justifiable biological and experimental uses of hESCs (developmental biology, drug screening and toxicity, models of the prenatal origin of certain cancers, disease models, potential future cell therapy), translation to clinical applications in cell replacement therapy requires prior solution of the ethical and technical barriers described above. The international scientific community is making rapid progress in understanding hESCs and iPS cells, particularly motivated by the enormous interest this field of work has raised. Nonetheless, great efforts will have to be made to provide an answer to still quite basic questions concerning their biology. Only then, we shall finally discover whether hESCs really are a safe tool, with great potential for regenerative medicine.

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## CHAPTER 16

# EPIGENETICS OF EMBRYONIC STEM CELLS

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**Abstract:** Understanding the molecular mechanisms involved in the control of cell differentiation during embryonic development is currently one of the main objectives of developmental biology. This knowledge will provide a basis for the development of new strategies in the field of regenerative medicine, one of the most promising weapons to fight many human diseases. Cell differentiation during embryonic development is controlled primarily by epigenetic factors, that is, mechanisms involved in the regulation of chromatin structure and gene expression. Here we describe the best known epigenetic modifications, and pathways, mainly focused on DNA methylation and histone modifications, and try to depict the state of art in our knowledge about epigenetic regulation of embryonic stem cell maintenance and differentiation.

## INTRODUCTION

A first definition of epigenetics was proposed by Conrad Waddington in 1942<sup>1</sup> as the study of how genotypes give rise to phenotypes through programmed changes during development. New concepts were subsequently added to this original definition: An epigenetic event would be something that affects gene expression without changing the nucleotide sequence, in a way that can be inherited through cell division and possibly through gamete formation.

Today, epigenetics refers to heritable changes in gene activity and expression, as well as to stable, long-term alterations in the transcriptional potential of a cell that are not necessarily heritable (<http://nihroadmap.nih.gov/epigenomics/index.asp>). Epigenetics in this sense would include all mechanisms for unfolding the genetic program in processes such as development, differentiation, stress response and pathological states. Epigenetic modifications are stable and at the same time plastic, as they can be modulated by

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cellular or environmental factors.<sup>2</sup> This nuance of plasticity is the most striking feature of epigenetics, as it enables elaboration of the genetic information and its integration with the environment. In other words, each gene is expressed in a defined space and time following the dictates of the epigenetic machinery that, by altering the physical structure of the genetic information, makes it readable or unreadable. Chromatin structure, like the genetic information itself, can be inherited by cell progeny, creating a new and stable level of information. Epigenetic mechanisms involve covalent chemical modification of DNA (methylation) or chromatin (histone modification), as well as other processes, mostly related to the former two, that regulate gene expression and chromatin structure (noncoding RNA, among others).

DNA, like a book, is organized into modules. All the epigenetic machinery can be seen as a complex system of enzymes or structural proteins. In response to a cell's internal and external status, these proteins are able to write the instructions in their own language for the accessibility of the basic book of each cell, i.e., the genome. Each page is represented by the nucleosome core particle. When associated with other components, higher-order nucleosomal structures are formed, like book chapters or sections. The epigenetic machinery is in charge of determining the accessibility of the pages to the readers of DNA, for example RNA polymerase. This ensures that the genetic information is stored, organized and read out in a correct spatio-temporal sequence during cell differentiation and organism development. The epigenetic code consists of a large number of small covalent modifications on DNA or histones. Among epigenetic proteins, we find enzymes that perform these covalent modifications, the "writers", as well as enzymes able to eliminate them, the "erasers". Finally, other proteins, the "readers" of the epigenetic code, recognize these modifications and join them to the effector function, opening or closing the chapter of the book. Variations introduced into nucleosome array structures by this machinery determine differences in chromatin compaction that correlate closely with "open" versus "closed" states, which in general co-incide with "active" versus "inactive" states of gene expression.

## CHROMATIN STRUCTURE

The basic repeating unit of chromatin is the nucleosome, first defined by Pierre Chambon in 1975.<sup>3</sup> In 1997, the 2.8 Å crystal structure of the nucleosome was resolved, revealing a ~147 bp double strand of DNA tightly wrapped in 1.7 left-handed superhelical turns around a core histone octamer (formed by two H2A-H2B dimers and one H3-H4 tetramer). Nucleosomes are joined by the linker DNA, together with a linker histone protein (H1). Specific interactions between nucleosomes determine the folding of a nucleosomal array (the primary structure of chromatin) into a 30 nm fiber (a secondary structure) and into large-scale configurations (tertiary structures) that constitute the entire chromosome.<sup>4</sup> Although the structure of the nucleosome core was further resolved at 1.9 Å resolution, the precise structure of the compacted 30 nm fiber remains unknown, as its compactness impedes visualization of the spatial location of individual nucleosomes and the path of the DNA.

In general, the close interactions between DNA and histone proteins in a nucleosome lead to a high degree of structural condensation that, by default, impedes gene transcription. Histones nonetheless have positively charged, unstructured tails that protrude from the nucleosome core and can be modified in many amino acid residues. There are

at least 30 possible histone modification sites for each nucleosome and eight types of modification (methylation, acetylation, phosphorylation, ubiquitylation, sumoylation, ADP-ribosylation, proline isomerization and biotinylation), some of which can occur in different configurations (for example, lysine can be mono-, di- or trimethylated); a considerable number of combinations are thus possible. The information contained in the different combinations of histone marks determines the functional state of the associated DNA; this language is often referred to as the histone code.<sup>5</sup>

Chromatin in eukaryotic cell nuclei is not uniformly organized, but rather contains distinguishable chromatin states: Euchromatin and heterochromatin. This simplification distinguishes two classes of chromatin, one found in a low condensation state that is “accessible” and one with a high degree of compaction that is “inaccessible” for transcription. A critical function of histone modifications is to establish and preserve these two states.<sup>6</sup> They are classified as repressing and activating, as correlated with gene repression or induction. Some modifications can nonetheless activate or repress in a context-dependent manner. The outcome of chromatin modification thus depends not on a single mark, but on the whole complex of modifications and their context.

## ACETYLATION

Acetylation of lysine 14 or 9 in histone H3 (H3K14, H3K9), and/or of H4K16 are generally associated with active gene transcription. Evidence supporting a positive link between acetylation of histone tails and transcriptional activity has long been established. Gene activation by histone acetylation has a biophysical explanation. The lysine side chain is positively charged and can bind tightly to the negatively charged DNA to form a closed chromatin structure that impedes access of transcription factors. Acetylation of lysine residues removes their positive charge and attenuates the charge interaction between histone tails and DNA. Indeed, it was demonstrated *in vitro* that acetylation of H4 at H4K16 has a negative effect on the formation of the 30 nm fiber and the generation of higher-order structures.<sup>7</sup> In addition to this simple mechanism, acetylated lysines also act as docking sites for other proteins that act as “readers”, mainly other chromatin-modifying enzymes and basal transcription machinery. One protein domain, termed the bromodomain, binds specifically to acetylated lysines,<sup>8</sup> facilitating transcriptional activation; this domain is often found in enzymes that help activate transcription, including SWI/SNF, an ATP-dependent chromatin-remodeling complex. Histones are acetylated in lysines by “writer” enzymes termed histone acetyl transferases (HAT), which transfer an acetyl group from the high-energy donor acetyl-coenzymeA to a lysine  $\epsilon$ -amino group.

Acetyltransferases are divided into three main families, GNAT, MYST, and CBP/p300. Many HAT are components of large multi-subunit complexes associated with transcription initiation, recruited to promoters by interaction with DNA-bound activator proteins. In general, these enzymes modify more than one lysine, and only a few HAT show some substrate selectivity. Histone acetylation is reversed by the “eraser” enzymatic action of the histone deacetylases (HDAC). HDAC are grouped into classes I, II, and III based on sequence homology to their yeast orthologues Rpd3, HdaI and Sir2, respectively,<sup>9</sup> and class IV, which has only one member (HDAC11). Classes I, II, and IV are referred to as “classical” HDAC and comprise 11 family members, whereas class III members are called sirtuins.<sup>10</sup> Classical HDAC and sirtuins differ in

their catalytic mechanisms. Classical HDAC are  $\text{Zn}^{2+}$ -dependent enzymes, harboring a catalytic pocket with a  $\text{Zn}^{2+}$  ion at its base that can be inhibited by  $\text{Zn}^{2+}$ -chelating compounds such as hydroxamic acids. In contrast, sirtuins have a mechanism of action that requires  $\text{NAD}^+$  as an essential cofactor.<sup>10</sup>

The sirtuins are so-named because of their homology to *Saccharomyces cerevisiae* Sir2, an enzyme implicated in epigenetic silencing of telomeres, ribosomal DNA and mating-type genes. The catalytic domain, highly conserved from Archaea to man, can carry out two types of reactions: Deacetylation and ADP ribosylation.  $\text{NAD}^+$  is involved in the transfer of electrons generated through intermediary metabolism pathways; the  $\text{NAD}^+/\text{NADH}$  ratio is therefore an important sensor of the redox state of the cell and of metabolism. Sir2-compacted chromatin is characterized by hypoacetylation of lysine residues in the N-terminal tails of histones H3 and H4 a very distinctive mark, hypoacetylation of H4K16, is a signature of Sir2 silencing.<sup>11</sup> In addition to epigenetic silencing, Sir2 has a role in DNA repair, recombination, and DNA replication.<sup>12</sup>

Seven sirtuin family proteins have been described in mammals, termed SirT1 to SirT7. SirT1, 2, 3 and 6 preferentially target histones, specifically H4K16 and H3K9.<sup>10</sup> Thus far, these are the only enzymes known to deacetylate acetyl-H4K16 in higher eukaryotes. SirT1, 2 and 3 also target nonhistone proteins, however, including various nuclear factors (SirT1), tubulin (SirT2) and mitochondrial targets (SirT3).<sup>13</sup> Although considered a nuclear protein, SirT1 localization varies with cell-type and differentiation stage; it can be exclusively cytoplasmic or present throughout the cell.

## PHOSPHORYLATION

Phosphorylation consists of adding a phosphate to a hydroxyl ( $\text{OH}^-$ ) group of serine or threonine residues, thereby adding a negative charge that leads to general decondensation of the chromatin fiber. The biophysical role of histone phosphorylation has not been demonstrated rigorously in vitro as for acetylated H4K16, but its role in mitosis, apoptosis and gametogenesis are suggestive of this mechanism.<sup>14</sup> Phosphorylated residues on histones are also bound by 14-3-3 protein, linking this modification to transcriptional activation.<sup>15</sup> As many cell signaling pathways are related to protein phosphorylation, this modification is an excellent link between activation of kinase signaling pathways in response to stimuli and gene expression. For instance, growth factor stimulation induces rapid phosphorylation of histone H3 at Ser 10 (H3S10) at c-Jun and c-Fos promoters, mediated by mitogen-and stress-activated protein kinases (MSK)1, MSK2 and RSK2.<sup>16</sup> H3S10 phosphorylation is detected after activation of  $\text{NF}\kappa\text{B}$ -regulated genes; in response to inflammatory cytokines,  $\text{I}\kappa\text{B}$  kinase- $\alpha$  ( $\text{IKK-}\alpha$ ) phosphorylates H3S10 at  $\text{NF}\kappa\text{B}$ -responsive promoters.<sup>17</sup> Other histone residues can also be phosphorylated: Threonine 11 of histone H3 (H3T11ph) is phosphorylated by protein-kinase-C-related kinase 1 (PRK1), leading to transcriptional regulation in response to stimulation with androgen receptor agonists.<sup>18</sup>

## METHYLATION

Methylation occurs on the functional group of lysines or arginines. This modification is functionally more complex than the others for several reasons: (1) within any histone,

multiple lysines or arginines can be modified; (2) individual lysine residues can be mono-, di-, or trimethylated; (3) arginine residues can be mono- or dimethylated, the latter in a symmetric or asymmetric fashion; (4) all the core histones can be methylated depending on physiological setting and finally; (5) methylation of distinct residues can have opposite consequences in gene activation. In the case of methylation, global charge of the residue is unaffected by the modification; the final effect is thus determined mainly by different “reader” proteins that link these marks with other effectors. Histone methylation is recognized by chromo-like domains of the Royal family (chromo, tudor, MBT) and the unrelated PHD domains. Proteins with a chromodomain, such as HP1, can bind specifically to methylated lysine. HP1 is a transcription-silencing protein that interacts with HDAC; its binding to methylated H3K9 results in histone deacetylation, eventually leading to gene silencing. H3K4 trimethylation is recognized by the chromodomain protein CHD1, which can further recruit HAT to activate target gene transcription.

These various methylation reactions are mediated by “writers” termed histone methyltransferases (HMT), enzymes that use an S-adenosyl methionine (SAM) high-energy methyl-donor to transfer the methyl group onto the histone residue. Each of these enzymes has its own activation pathways and its own specificity.

Arginine methylation is catalyzed by the protein arginine N-methyltransferase (PRMT) family of enzymes. Arginine can be monomethylated, symmetrically dimethylated and asymmetrically dimethylated, with each combination showing potentially different functional consequences.<sup>19</sup> Ten mammalian PRMT (PRMT1-10) have been identified to date. CARM1, sometimes referred to as PRMT4, can methylate histone H3 at arginines 2, 17, and 26 (H3R2, H3R17, H3R26) and enhances transcriptional activation driven by nuclear receptors, serving as a co-activator.<sup>20</sup> PRMT1 is also required for transcriptional activation by nuclear receptors, methylating arginine 3 of histone H4 (H4R3).<sup>21</sup> Several co-activators with histone-modifying activities can co-operate synergistically; e.g., CARM1 activity can co-operate with CBP, pCAF, and p300, all involved in histone acetylation. This protein family also targets many nonhistone proteins.<sup>22</sup>

Lysine methylation is operated by “writers” called lysine methyltransferases (KMT), typically specific in their histone targets; their output can be activation or repression of transcription.<sup>6</sup> Almost all histone KMT characterized to date have a SET domain (named after *Drosophila melanogaster* Su(var)3-9, Enhancer of zeste (E(z)), and trithorax (trx)). SET domain-containing enzymes can catalyze methylation of specific lysines on histones H3 and H4. DOT1L, the mammalian orthologue of the yeast histone methyltransferase Dot1, differs from other histone KMT as it lacks a SET domain and cannot modify free histones, but rather requires a nucleosomal substrate.<sup>23</sup> DOT1L catalyzes mono-, di- and trimethylation of histone H3K79, a residue located in the nucleosome core. Although different histone KMT can share substrate specificity, it is likely that each enzyme can regulate different genes or cell processes.

Three methylation sites on histones are implicated in activation of transcription, H3K4, H3K36 and H3K79.<sup>6,24</sup> H3K4, both di- and trimethylated, is enriched at actively transcribed genes.<sup>25</sup> Whereas the H3K4me2 modification appears to be distributed throughout the body of active genes, however, H3K4me3 modification is localized specifically at the 5' end of these genes.<sup>26</sup> The SET domain-containing protein MLL is the mammalian H3K4-specific KMT and is detected only in a multi-subunit complex with the conserved structural components RbBP5, Ash2L, and WDR5. MLL1 localizes with RNA polymerase II to the 5' end of 90% of actively transcribed genes, where H3K4me3 is also found.<sup>27</sup> H3K36 methylation seems to facilitate transcription elongation. H3K36me2 and H3K36me3 are in fact enriched



at the 3' ends of transcribed genes.<sup>26</sup> H3K79 methylation correlates with transcriptional activation,<sup>28</sup> and DOT1L in mammals preferentially occupies the proximal transcribed region of active genes and correlates with the presence of H3K79me2 and H3K79me3.<sup>29</sup>

Three methylation sites on histones are linked to transcriptional repression: H3K9, H3K27 and H4K20.<sup>6,24</sup> Methylation at H3K9 is implicated in the silencing of euchromatic genes and in the formation of silent heterochromatin domains. Repression involves recruitment of H3K9-specific KMT (SUV39h1 and SUV39h2) followed by HP1 to the promoter of repressed genes. H3K27 methylation is implicated in several silencing phenomena, including *HOX* gene silencing, inactivation of the X chromosome in females, and genomic imprinting.<sup>30</sup> At the core of this silencing system is the Polycomb group (PcG) proteins, usually found in multiprotein complexes termed Polycomb repressive complexes (PRC). PRC2 contains EZH2, EED, SUZ12 and RbAp48, whereas the PRC1 complex consists of >10 subunits, including BMI-1 and the Polycomb proteins (CBX2, CBX4, CBX7, CBX8), (HPH1-3, RING1-2 and SCML).<sup>31</sup> Many PcG target genes are involved in developmental patterning, morphogenesis, and organogenesis.<sup>32,33</sup> Functionally, EZH2 is the catalytically active component of PRC2, acting as an H3K27-specific KMT.<sup>30</sup> PRC can inhibit transcription by preventing ATP-dependent nucleosome remodeling by the SWI/SNF complex, as well as by directly blocking the transcription initiation machinery.<sup>34</sup> PRC2 shows KMT activity towards H1K26 in vitro,<sup>35</sup> tethering HP1 to chromatin<sup>36</sup> and recruits DNA methyltransferases (DNMT) to selected target genes.<sup>37</sup> H4K20 is monomethylated by PR-Set7/Set8, and trimethylated by Suv4-20h1 and Suv4-20h2.<sup>38</sup>

Several families of lysine demethylases (KDM) have been identified, which eliminate methyl marks associated with gene repression or activation; KDM usually form part of large multiprotein complexes that synergize with HDAC, KMT and nuclear receptors to control developmental and transcriptional programs. Two types of demethylase domain have been reported thus far, with distinct catalytic reactions: The LSD1 domain and the Jumonji C (JmjC) domain.<sup>39</sup> LSD1 can function as a KDM specific both for H3K4me2/me1 and H3K9me1/me2, and for nonhistone substrates such as p53. Enzyme specificity is determined partly by its association with different complexes, thereby allowing it to participate in transcriptional regulation, heterochromatin spreading and stress-induced responses.<sup>39</sup> At difference from LSD1, the JmjC domain-driven demethylase reaction allows demethylation of a trimethylated histone tail. There are 27 JmjC domain proteins within the human genome, 15 of which demethylate specific lysines or arginines in the H3 tail.<sup>40</sup>

## UBIQUITYLATION

This very large modification has been found on H2A (K119) and H2B (K120 in human and K123 in yeast). Ubiquitylation of H2AK119 is mediated by the Bmi/Ring1A protein found in human PRC1 and is associated with transcriptional repression.<sup>41</sup> In contrast, H2BK120 ubiquitylation is mediated by human RNF20/RNF40 and UbcH6 and activates transcription.<sup>42</sup> The mechanism of ubiquitin function is still unclear; it is likely to recruit additional factors to chromatin, but given its large size, it might also act to maintain chromatin open by a “wedging” process.

## SUMOYLATION

Sumoylation is another very large modification, and shows some similarity to ubiquitylation. This modification takes place on all four core histones, and specific sites have been identified on H4, H2A, and H2B.<sup>43</sup> Sumoylation antagonizes both acetylation and ubiquitylation, which occur on the same lysine residue; consequently, this modification is repressive for transcription in yeast.

## ADP RIBOSYLATION

This histone modification can be divided into two major groups: Mono- and poly-ADP ribosylation, mediated by MART (mono-ADP-ribosyltransferases) or PARP (poly-ADP-ribose polymerases), respectively.<sup>44</sup> Although the function of these enzymes has often been linked to transcription, evidence that the catalytic activity is involved is lacking.

## PROLINE ISOMERIZATION

Prolines exist in either a cis or a trans conformation. These conformational changes can severely distort the polypeptide backbone. An enzyme, FPR4, isomerizes different prolines in the H3 tail.<sup>45</sup> Isomerization of H3P38 regulates H3K36 methylation levels, as the appropriate proline isomer is likely to be necessary for H3K36 recognition and methylation by the Set2 KMT, as well as for its demethylation. The catalytic cleft of the JMJD2 demethylase is very deep and may necessitate a bend in the polypeptide (mediated by proline isomerization) to accommodate the methyl group at H3K36.

## HISTONE CROSSTALK

The existence of several modifications within a short stretch of the same histone tail makes crosstalk likely. A number mechanisms have been reported: (1) histone modifications cannot co-exist on the same residue and are mutually exclusive, as is the case for acetyl- and methyl-H3K9;<sup>46</sup> (2) protein binding might be disrupted by an adjacent modification (e.g., phosphorylation of serine 10 inhibits HP1 binding to methylated H3K9);<sup>47</sup> (3) catalytic activity of an enzyme can be compromised by modification of its substrate recognition site (H3P38 isomerization affects H3K36 methylation by SET2);<sup>45</sup> (4) an enzyme could recognize its substrate more effectively in the context of a second modification, as is the case of the GCN5 acetyltransferase, whose action on H3 is enhanced by phospho-H3S10;<sup>48</sup> and (5) modifications on different histones can affect one another (ubiquitylation of H2B is necessary for methylation of H3K4me3 and H3K79me2).<sup>49</sup>

## HISTONE VARIANTS

The vast majority of histones in eukaryotic cells are expressed during the S phase and deposited on nucleosomes during DNA replication. Variants of histones H3 and H2A are nonetheless synthesized throughout the cell cycle and deposited in a replication-independent manner, conferring specialized function on nucleosomes. Centromere protein A (CENP-A) is an H3 variant with an essential role in the assembly of centromeric nucleosomes.<sup>50</sup> Its N-terminal tail is very different from that of H3, presenting the opportunity for alternative posttranslational modifications. H3.3 is deposited primarily in transcriptionally active chromatin and gene regulatory sites.<sup>51</sup> The H2A variant H2AZ is implicated in both gene activation and repression. It is localized in small regions flanking transcription start sites and larger regions proximal to telomeres or centric heterochromatin.<sup>26,52</sup> In ESC, it binds to promoters of developmentally important genes, as does the PcG protein Suz12.<sup>53</sup> H2AX has a unique C-terminal extension, important for its phosphorylation, which is important in DNA repair.<sup>54</sup> MacroH2A also has an extra C-terminal motif that binds O-acetyl-ADP-ribose and replaces H2A in nucleosomes of the inactive X chromosome in female mammals,<sup>55</sup> while also regulating key developmental genes in human male pluripotent cell autosomes.<sup>56</sup> Finally, deposition of the H2ABbd (Barr body-deficient) variant correlates with transcriptionally active chromatin.<sup>57</sup>

## ATP-DEPENDENT CHROMATIN REMODELING

Another group of chromatin regulators, referred to as ATP-dependent chromatin remodeling complexes, uses ATP hydrolysis to alter histone-DNA contacts.<sup>58</sup> They share a common ATPase subunit of the SNF2 superfamily of DNA helicase/ATPase. ATP-dependent nucleosome remodeling factors are classed into subfamilies, depending on the presence of other domains within the ATPase-containing subunit. The four main subfamilies characterized to date are the SWI/SNF, ISWI, CHD, and INO80 subfamilies.<sup>58</sup> These complexes mediate different nucleosome remodeling activities, ranging from subtle shifting of nucleosome positions to histone exchange or complete ejection of nucleosomes. In essence, all unwrap DNA segments from the nucleosome surface and translocate them through the nucleosomes.<sup>59</sup> These complexes are involved in many fundamental processes such as transcription, DNA repair, DNA replication and chromosome structure maintenance, and are precisely regulated at many levels. They are often targeted by specific histone modifications and transcriptional regulators, as is the case of CHD1, which binds methylated H3K4 through its tandem chromodomain.<sup>60</sup>

## DNA METHYLATION

DNA methylation is the simplest and perhaps best-studied epigenetic modification. In mammals, it generally consists of the addition of a methyl group to the 5-carbon of a cytosine followed by a guanine (CpG) in the DNA sequence. Throughout the mammalian genome, the CpG dinucleotide is found at a frequency much lower than expected, probably because methylation of cytosine leads to its conversion into thymine upon deamination. It nevertheless reaches a frequency close to that predicted in sequences

that span the 5' end of many genes, regions termed CpG islands; a CpG island is thus defined by the occurrence of this dinucleotide. CpG islands are usually found in the regulatory regions of vertebrate housekeeping genes; they are often protected from methylation, enabling constitutive expression of these genes. During development, a subset of CpG islands is subjected to dynamic methylation modifications linked to tissue differentiation and formation.<sup>61</sup> Once differentiation is complete, tissue-specific methylation is established in each cell-type and is generally maintained throughout the cell's life. Promoter methylation accounts for only a small part of overall genome methylation; roughly 70% of all CpG are methylated and the majority are located in repetitive sequences such as endogenous retroviruses, LINE1 and Alu elements, most of which are derived from transposable elements.<sup>62</sup> Methylation maintains these sequences silent, making the event of amplification and new insertion in the genome extremely rare. It has in fact been proposed that DNA methylation might have evolved as a genome defense system to prevent the chromosomal instability, translocations and gene disruption caused by re-activation of these transposable DNA sequences.<sup>62</sup>

At least two additional genetic mechanisms, genomic imprinting and X-chromosome inactivation, rely on DNA methylation in normal cells. Genomic imprinting occurs in some genes whose expression is always restricted to either the maternal or the paternal allele; imprinting requires DNA methylation at one of the two parental alleles to ensure monoallelic expression. A similar gene-dosage reduction is involved in X-inactivation in females.<sup>63</sup>

The “writers” of these modifications are members of the DNA methyltransferase family of enzymes. DNMT1 is the most abundant methyltransferase in somatic cells, it localizes to replication foci and interacts with the proliferating cell nuclear antigen (PCNA).<sup>64</sup> When a new DNA strand is synthesized, the methyl-CpG site is copied to an antisense CpG on the other strand, creating a hemi-methylated site. DNMT1 specifically recognizes these hemi-methylated CpG<sup>65</sup> and transfers a methyl group to the unmethylated cytosine ring; in this way, methylation can be transmitted to both daughter cells. These features explain the stability of the modification, which enables it to be inherited in cell division. DNMT1 is necessary for correct embryonic development, imprinting and X-inactivation.<sup>66</sup> DNMT3A and DNMT3B are required for the wave of de novo methylation that occurs in the genome following embryo implantation and of newly integrated retroviral sequences in mouse ESC.<sup>67</sup> Moreover, *Dnmt3a* and *Dnmt3b* knockout (KO) mice show severe developmental defects that cause death at newborn and embryonic stages, respectively. *DNMT3B* mutations underlie ICF syndrome, a rare disorder characterized by immunodeficiency, centromeric instability, and facial abnormalities.<sup>68</sup> These observations, coupled with in vitro data indicating that the DNMT3 enzymes show equal preference for hemi- and unmethylated DNA substrates, have led to the term “de novo methyltransferases” to describe DNMT3.<sup>67</sup> The fourth member of the family, DNMT2, has to date no clearly ascribed function in DNA methylation,<sup>69</sup> but transfer RNA methyltransferase activity has been reported.

The “eraser” responsible for reversion of this modification has not yet been identified univocally in animals (although it is described for plants);<sup>70</sup> some candidates have nonetheless been proposed. Although there are numerous exceptions, most genome methylation is not maintained during meiosis, and gamete formation.<sup>71</sup> The entire genome undergoes global demethylation in gametes and methylation is subsequently re-established in early embryonic stages (see section on epigenetic events in pre-implantation).

DNA methylation is linked to transcriptional silencing. Early experiments showed that DNA methylation leads to formation of a compacted, nuclease-resistant chromatin state.<sup>72</sup> It was proposed that the methyl group could interfere with protein binding to its cognate DNA sequence, such as a transcription factor. In most cases, however, methyl-CpG attracts rather than repels proteins; the “readers” for this modification are a family of proteins that contain the methyl-binding domain (MBD). These proteins link the CpG methyl group to complex chromatin remodeling machinery that turns off transcription and locks the chromatin in a condensed state. Methyl-CpG binding protein 2 (MECP2) was the first to be described, and later gained in interest, as it is mutated in individuals with Rett syndrome.<sup>73</sup> MECP2 has two domains: The MBD recognizes a symmetrically methylated-CpG dinucleotide, and a transcriptional repression domain (TRD) interacts with several other regulatory proteins with HDAC activity.<sup>74</sup> Other MBD-containing proteins (MBD1, MBD2, MBD3 and MBD4) have also been described.<sup>75</sup> Except for MBD4, which is involved in repairing DNA mismatches, the MBD family members belong to similar HDAC-containing complexes. All MBD proteins interact specifically with methylated DNA except MBD3 (targeted to methylated DNA through association with MBD2). An unrelated protein, Kaiso, also binds methylated DNA and interacts with an HDAC complex, N-CoR.<sup>76</sup> Other levels of crosstalk between DNA methylation and histone modifications are reported: DNMT and MBD proteins can recruit the H3K9 methylase SUV3-9h1;<sup>77</sup> DNMT also interact with KMT such as EZH2 in the PRC2 complex, as described above, and with HDAC complexes.<sup>78</sup>

## THE MAMMALIAN EMBRYO

Development is considered the process that, through progressive changes, leads to formation of a complete organism from a single cell, and fulfils two major objectives: It generates cell diversity and order within each generation, and ensures the continuity of life from one generation to the next.

After fertilization of the mammalian egg, the derived zygote, the first cell of a new organism, begins to divide. These first symmetric cleavages give rise to 2, 4, and 8 virtually identical cells, blastomeres; these are classically considered the only totipotent cells, in the sense that each has the potential to give rise to any of the differentiated cells of the embryonic and extraembryonic tissues. Cells in the compact 8-cell embryo divide again to form a 16-cell morula, constituted by a small group of internal cells surrounded by a larger group of external cells.<sup>79</sup> The offspring of external cells give rise to the trophoblast cells, producing the chorion, the embryonic portion of the placenta. The embryo proper is derived from descendants of the inner cells of the 16-cell stage, supplemented by cells dividing from the trophoblast during the transition to the 32-cell stage.<sup>80</sup> These cells generate the inner cell mass (ICM), which will generate the embryo and its associated yolk sac, allantois, and amnion. By the 64-cell stage, the ICM (approximately 13 cells) and the trophoblast cells have become separate layers, and neither contributes cells to the other group. Thus, the distinction between trophoblast and ICM blastomeres represents the first differentiation event in mammalian development. In the morula, during a process called cavitation, the trophoblast cells secrete fluid to create a cavity known as the blastocoel. The ICM is then positioned on one side of the ring of trophoblast cells; the resulting structure is termed blastocyst. Isolation of the

ICM at this stage and its propagation in vitro in appropriate conditions leads to the derivation of embryonic stem cell (ESC) lines. These cells are defined as pluripotent since, although they can form any embryonic cell lineage, they have already passed the first differentiation step.

After implantation in the uterine wall, the first segregation of cells within the ICM results in formation of hypoblast and epiblast. Hypoblast cells delaminate from the ICM to line the blastocoel, where they give rise to the extraembryonic endoderm, which forms the yolk sac. The remaining ICM tissue above the hypoblast is now referred to as the epiblast. Epiblast cells also cover the entire internal surface of the cavity they face, the amniotic cavity. Once the amnion lining is complete, it fills with amniotic fluid. The epiblast alone is responsible for embryo formation; however, the hypoblast develops into a part of the extraembryonic appendages.

The bilaminar germ disk evolves to a trilaminar embryo. By day 17 in the human embryo, thickening of the embryonic disc primitive streak is observed, due to cell proliferation and migration towards the median line along the rostro-caudal axis. After day 19, the primitive streak grows through cell addition at its caudal end. At the anterior end, a groove begins to form (primitive groove). Epiblast cells migrating through the primitive streak, between the two germinal layers, determine the formation of three new embryonic layers: The dorsal-lying cell layer facing the amniotic cavity (ectoderm); ventrally-lying cells that form the endoderm, which replaces the hypoblast, and cells residing between the last two, forming the mesoderm.<sup>81</sup> At this stage, the embryonic disk is oval-shaped and the ectoderm is bathed in amniotic fluid. In a few days, many different specialized cells form; the embryo itself and extraembryonic tissues are thus generated from a few identical cells.

From this point onward, several decisions must be made, not only regarding the fate of each cell to be able to establish a defined differentiation program, but also regarding more complex patterning mechanisms that define the structure of the entire organism. These mechanisms include definition of the three axes (anterior-posterior, dorsal-ventral, and left-right), organ formation, and integration of complex systems-like the cardiovascular, musculoskeletal, and nervous systems. These processes involve multilevel organization of the molecular information, which continues to unfold as long as the embryo develops and system complexity increases. This is achieved through several mechanisms, including cell-cell communication, signaling routes, and gene expression programs that are finely regulated in space and time.

An example of a complex genetic mechanism involved in embryo patterning is the anterior-posterior polarity specification by expression of *Hox* genes, homologues to the homeotic gene complex (*Hom-C*) of the fruit fly. Mouse and human genomes contain four copies of the *Hox* complex per haploid set, located on four different chromosomes (*Hoxa* through *Hoxd* in the mouse, *HOXA* through *HOXD* in man).<sup>82</sup> They are arranged in the same general order as their expression pattern along the anterior-posterior axis, with the most 3' gene required for producing the most anterior structures, and the most 5' gene specifying the development of the posterior abdomen. *Hox* gene expression can be seen along the dorsal axis (in the neural tube, neural crest, paraxial mesoderm and surface ectoderm) from the anterior boundary of the hindbrain through the tail. Different body regions from the midbrain through the tail are characterized by various constellations of *Hox* gene expression, and these expression patterns create a code whereby certain *Hox* gene combinations specify a particular region of the anterior-posterior axis.<sup>83</sup>



## EMBRYONIC STEM CELLS, IN VITRO DIFFERENTIATION AND APPLICATIONS

In 1998, two laboratories announced that they had derived human embryonic stem cells (hESC).<sup>84,85</sup> These cells were derived either from ICM of embryos that could not be implanted into in vitro fertilization patients, or from germ cells from spontaneously aborted fetuses. In both instances, the hESC were pluripotent, since they were able to differentiate in culture to form more restricted stem cells or differentiated cells; when injected into immunodeficient mice, they produced a teratoma containing many tissue types. Since then, hESC have become a powerful model with which to understand the biology of the first developmental steps. Many efforts have been made to define the best conditions in which to maintain these cells in culture<sup>86</sup> and to induce specific differentiation. In many cases, the goal is to optimize protocols for application in regenerative medicine.

ES cells are currently generated by whole or partial embryo culture, or by immunosurgery, the isolation and culture of ICM cells after enzymatic removal of the *zona pellucida* and complement-mediated disruption of trophoectoderm.<sup>87</sup> Establishment of ESC lines depends on many factors, including culture conditions, feeder cell growth surface, culture medium composition and even genetic background.<sup>88</sup>

Induced pluripotent stem (iPS) cells represent a novel, attractive model system for the study of epigenetic mechanisms that operate during preimplantation development. By overexpressing key embryonic transcription factors (OCT4/POU5F1 and SOX2) along with the transcriptional activators KLF4 and cMYC, the somatic epigenome is erased and its embryonic counterpart established.<sup>89,90</sup> iPS cells have been derived by introducing different combinations of the above-cited and other factors using viral- or plasmid-based gene transfer methods,<sup>91</sup> sometimes in conjunction with small molecules,<sup>92</sup> or by direct introduction of recombinant proteins into cells.<sup>93</sup> Many different cell types—differentiated and partially undifferentiated, both from healthy donors and from patients—have also been used as a starting point.<sup>94,95</sup> The relevance of iPS cells as a model system for embryonic development was corroborated by the successful derivation of cloned mice via tetraploid complementation.<sup>96</sup>

The main concern for implementation of the data generated in ES and iPS cells, both in basic science and translational application, are possible alterations derived from the prolonged in vitro culture necessary for cell expansion to numbers sufficient for biochemical analysis. These extended culture regimens frequently lead to aneuploidy, and are likely to induce aberrant mechanisms that increase cell survival in culture, but are unrelated to the uterine environment. Comparison of epigenetic marks in ICM cells and cells from an established ESC line demonstrated loss of these marks in ESC.<sup>97</sup> Nonetheless, this discrepancy might not only be the result of culture adaptation of clonal ESC, but could also reflect heterogeneity in the ICM cell pool and account for the low efficiencies encountered during targeted differentiation of ESC.

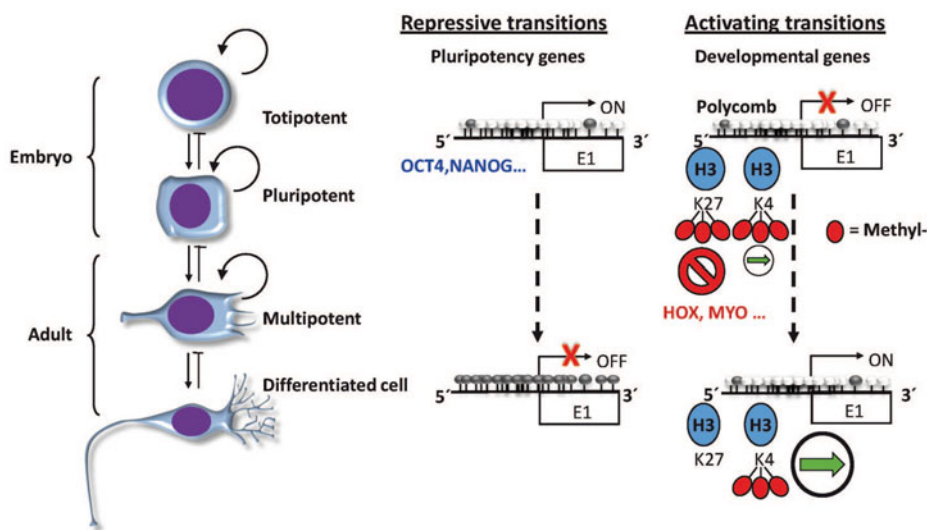
The hope is that hESC can be used to produce new functional cells for pathological situations in which cell function is compromised. First attempts at stem cell-based therapy were tested in mouse, where ES-derived glial stem cells were transplanted into mice with a genetic deficiency in glial function and cured the defect;<sup>98</sup> ES-derived neural stem cells were able to divide and differentiate into functional neurons when injected into a damaged rodent nervous system.<sup>99</sup> Since these demonstrations, many applications in regenerative medicine have been tested worldwide. Research in this field has focused on overcoming the main problems for clinical application, which include differentiation



towards the desired lineages,<sup>100,101</sup> efficient differentiation to eliminate the potential tumorigenicity of these cells,<sup>102</sup> and overcoming host rejection.<sup>103,104</sup> The possibility of a model of in vitro differentiated tissues of many kinds is also appealing for the world of drug discovery and validation.<sup>105</sup>

## EPIGENETIC MECHANISMS IN EMBRYONIC STEM CELL DIFFERENTIATION

Early in development, the first zygote-derived cells must perform two different functions. One is self-renewal, which is the ability to expand the cell population that maintains a certain degree of potency until a definite developmental stage is reached; the other is totipotency (for the zygote and first cell divisions; pluripotency for the ICM and derived cultured ES cells), the ability to maintain the potential to give rise to any differentiated cell of the adult body. To achieve totipotency, germ cell epigenetic states must first be reset, and the zygotic genome must then be activated to allow expression of appropriate genes in subsequent development.<sup>2</sup> When a differentiation step is to be initiated, each cell must be able to modify its gene expression program to change its phenotype. To simplify a complex scenario that has only begun to be described, a stem cell appears to have a globally open chromatin structure that keeps pluripotency maintenance genes active and differentiation genes silenced, in a reversible, highly plastic way. Following differentiation towards a definite lineage, a stem cell must (a) downregulate pluripotency genes definitively (Fig. 1, left),<sup>106</sup> (b) activate expression of genes for the necessary

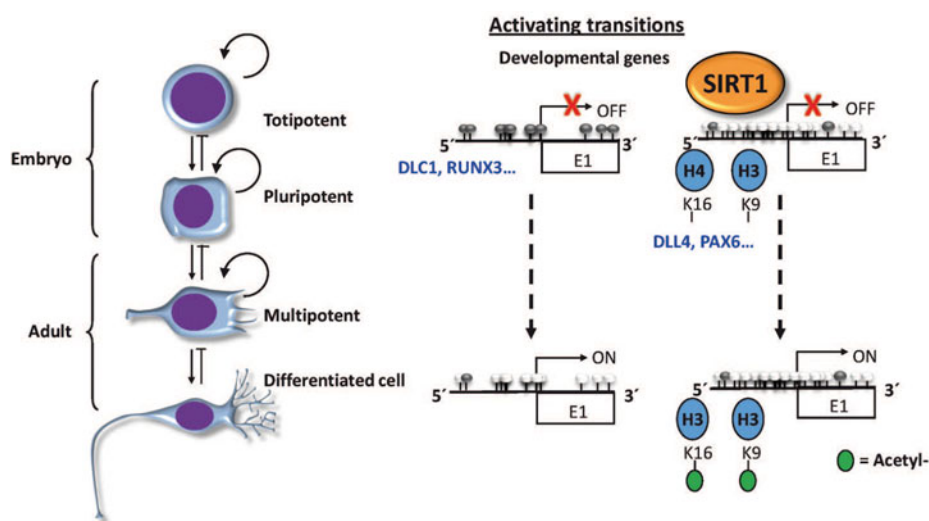


**Figure 1.** Epigenetic regulation of hESC differentiation. hESC differentiation involves inactivation of pluripotency genes (repressive transitions; left) and activation of developmental genes (activating transitions; right). Many pluripotency genes are inactivated during differentiation through promoter hypermethylation (left). The best-known mechanism involved in developmental gene silencing in hESC is bivalent mark/Polycomb-mediated inactivation, whose resolution is responsible for developmental gene activation during differentiation (right).

differentiation phenotype (Fig. 1 right and Fig. 2), and (c) lock differentiation genes for other lineages into a silent state (see following sections). Moreover, these processes must be precisely regulated in space and time. All of these skills are conferred on a stem cell mainly by the action of the epigenetic machinery.

## EPIGENETIC EVENTS IN PRE-IMPLANTATION

Two cycles of DNA methylation characterize gametogenesis and pre-implantation development in mammals. In the first week of embryonic development, primordial germ cells arise in the extraembryonic region posterior to the primitive streak, and are designated to regain the capacity to form a new organism.<sup>107</sup> Primordial germ cell differentiation involves a wave of genomic demethylation, which resets differentially methylated loci, including imprinted and allele-specific regions, followed a few days later by methylation.<sup>108</sup> Mature sperm and oocytes remain highly methylated until fertilization, after which a second cycle of demethylation and gradual remethylation of parental genomes occurs. The paternal genome is rapidly demethylated by an active mechanism, since it occurs in the absence of replication or transcription.<sup>109</sup> Various DNA repair enzymes are proposed to carry out active demethylation in mammals,<sup>110</sup> and a role was recently suggested for the *de novo* methyltransferases DNMT3A and DNMT3B. Co-incident with the first embryonic cleavage divisions, the zygotic genome is gradually demethylated until the morula stage. This probably occurs through a passive mechanism, whereby newly replicated DNA strands fail to be methylated by DNMT1, resulting in an overall increase in unmethylated DNA. This is due to sex-specific splicing events that control DNMT1 activity in early



**Figure 2.** Two recently described activating mechanisms for developmental genes. Some “late differentiation” genes are hypermethylated in hESC and are activated through promoter demethylation during differentiation (left). Other genes are maintained silent in hESC, in part through SirT1-mediated promoter deacetylation and are expressed later in development, modulated by SirT1 downregulation and promoter acetylation (right).

embryos by regulating its translation or nucleo-cytoplasmic localization.<sup>111</sup> De novo DNA methylation begins after blastocyst formation; cells that comprise the ICM show higher methylation levels than those of the trophectoderm, probably due to differential regulation of DNMT3B expression, decreased in extra-embryonic tissues and increased in ICM-derived cells.<sup>112</sup> Histone modification is also regulated specifically during pre-implantation development.<sup>113</sup>

## EPIGENETICS OF EMBRYONIC STEM CELLS

ESC are thought to be an adaptation of cell culture conditions, because the properties of the ICM are transient in nature, whereas cultured ESC divide indefinitely without losing pluripotency. Epigenetic changes during ESC differentiation in culture might nonetheless provide an informative model for events that regulate early embryonic development.

ESC must retain pluripotency while proliferating rapidly in culture. A group of transcriptional regulatory proteins are critical for pluripotency maintenance of both ESC and the ICM. The POU5F1 (Oct4) and NANOG transcription factors are necessary for correct ICM and ESC differentiation and, in conjunction with the high-mobility-group SOX2 protein, directly control the expression of genes important for both pluripotency and developmental pathways.<sup>114</sup> In mESC, Nanog and Oct4 seem to be associated to each other, and recruit specific repression complexes to their target genes, including elements of the NuRD and Sin3A complexes. A previously undescribed Hdac1/2- and Mta1/2-containing complex, defined as NODE (Nanog and Oct4-associated deacetylase), might be the main effector of gene repression mediated by these factors.<sup>115</sup> Detailed analysis of the pluripotency network showed that Nanog plays the leader role for generation and maintenance of the ground state of pluripotency, both in vivo (in the epiblast) and in vitro (in hESC and iPS generation).

In addition to regulating a network of developmentally important genes in ESC, OCT4 positively regulates expression of the H3K9me2 and H3K9me3 demethylases KDM3A (JMJD1A) and KDM4C (JMJD2C).<sup>116</sup> Undifferentiated ESC appear to have a more open chromatin structure than differentiated cells and are depleted of H3K9me3.<sup>117</sup> Activation of H3K9-specific demethylases by the core pluripotency regulatory machinery would cause an overall reduction in H3K9me3, directly linking the ESC pluripotency circuitry to the formation of an open chromatin structure.<sup>116</sup> In addition to posttranslational chromatin modification, CDH1 remodeling of chromatin structure also performs its function by maintaining an open chromatin state in ESC, localizing mainly in active gene promoters. Downregulation of this factor leads to heterochromatin accumulation and loss of pluripotency, as cells can no longer give rise to certain lineages.<sup>118</sup>

As stated above, ESC must maintain silencing of differentiation genes. This is achieved at least in part through the creation of bivalent chromatin domains. These domains are termed “bivalent”, as they contain overlapping regions of the transcriptionally permissive histone modification trimethyl-H3K4 and the transcription silencing mark trimethyl-H3K27, mediated respectively by the mixed-lineage leukemia (MLL) complex and the EZH2 component of PRC2.<sup>119</sup> Bivalent domains maintain silencing of developmentally important genes—primarily transcription factors such as the *Hox* and other tissue-specific genes—while simultaneously keeping them poised for either repression or activation, depending on the developmental lineage for which the ESC

are destined.<sup>119</sup> In essence, this bivalent configuration is thought to enable retention of developmental plasticity by these genes.

Histone KDM are responsible for the resolution of bivalent chromatin domains.<sup>39</sup> For example, *Hox* gene expression is strongly upregulated following ES differentiation, and H3K27me3 demethylase KDM6A (formerly UTX) binding at HOX promoters is increased concomitantly; this leads to a reduction in H3K27me2 and H3K27me3 and dissociation of PRC2.<sup>120</sup> Strikingly, Jarid2 (JMJ), a H3K27 demethylase, is physically associated to the PRC2 complex in stem cells, and is required for efficient PRC2 binding to its target, but also inhibits K27 methylation, thus acting as an internal modulator of PRC2 during development.<sup>121</sup> Other bivalent domain-marked genes require stable repression within specific developmental pathways. This is achieved by the tandem action of PRC2 and the H3K4me3 demethylase KDM5A (RBP2; also known as JARID1A), whose recruitment results in increased H3K27me3 and decreased H3K4me3 at these promoters.<sup>122</sup>

There are other important modifications in the first stages of developing embryo. In the mouse embryo, H3R26 and R17 methylation levels are heterogeneously distributed as early as four-cell stage blastomeres; cells with higher levels of this mark are more likely to contribute to ICM, while lower levels target cells to mural trophoectoderm. This observation indicates that the only real totipotent cell might in fact be the zygote. CARM1, the enzyme responsible for these modifications, thus seems to be necessary for blastomere and ICM cell pluripotency; CARM1-overexpressing cells strongly upregulate *Nanog* and *Sox2*.<sup>123</sup> CARM1 is also needed for ESC self-renewal and pluripotency, as CARM1 knockdown downregulates pluripotency genes, leading to differentiation. CARM1 associates with *Pou5f1* and *Sox2* promoters, which have detectable levels of R17/26 histone H3 methylation.<sup>124</sup>

Histone acetylation is also implicated in gene regulation in stem cells. The class III HDAC SirT1 was thought to be implicated in development, as mutant mice for this protein die pre- or perinatally; survivors are smaller and sterile, have eyelid-opening problems, chronic lung infection, pancreatic atrophy,<sup>125</sup> and developmental defects of the retina and heart. These data suggest that SirT1 acts in early development, in a role that is not likely to be essential, since mice pass through early embryonic stages and in some cases reach adulthood; SirT1 might have a modulatory effect on basic developmental processes. The main biological form of SirT1 is a homotrimer complex of ~350 kDa,<sup>126</sup> but in stem cells it forms the unusual PcG-related complex PRC4, functionally similar to PRC2.<sup>127</sup>

A model for SirT1 action in chromatin remodeling postulates that, following the arrival of SirT1 to chromatin, it deacetylates H4K16 and H3K9 and recruits histone H1 directly, which it deacetylates at K26.<sup>126</sup> H1K26 can be then methylated by EZH2,<sup>128</sup> which co-exists with SirT1 in the PRC4 complex. Thereafter, HP1 protein binds to dimethylated H1K26 through the HP1 chromodomain.<sup>36</sup> At the same time, the active chromatin mark dimethyl-H3K79 is lost over a stretch of a few kilobases from the promoter regions.<sup>28</sup> Lastly, SirT1 promotes the establishment of heterochromatin marks, specifically H3K9me3 and H4K20me, in the former case by interaction with Suv39h1.<sup>129</sup> SirT1 induces Suv39h1-dependent H3K9 trimethylation by deacetylation of H3K9 and recruitment of Suv39h1 through its N-terminal region. Moreover, its binding to Suv39h1 increases its methyltransferase activity in vitro and in vivo, probably through a conformational change. Finally, SirT1 deacetylates K266 of Suv39h1, a residue located in the catalytic SET domain, rendering the enzyme more active.

SirT1 was recently implicated in the first steps of hESC differentiation. SirT1 is strongly expressed in mouse and human ESC and is gradually downregulated during differentiation.

This regulation of SirT1 levels appears to be mediated by various mechanisms involving mRNA stabilization by arginine methylation of a well-known RNA binding protein, HuR. In hESC, SirT1 directly binds and represses promoters of a subset of developmental genes by deacetylating histones, and its downregulation during differentiation modulates the correct spatio-temporal activation of these genes.

Compared to differentiated cells and cancer cells, hESC possess a unique DNA methylation signature,<sup>130-132</sup> supporting the concept that a specific DNA methylation pattern could contribute to the pluripotent state. A genome-wide DNA methylation study in several somatic cell types and sperm was the first to highlight a relationship between promoter DNA methylation and promoter activity, which apparently depends on promoter CpG content.<sup>133</sup> Promoters with low CpG levels showed no correlation between their activity (defined by RNA polymerase II occupancy, not in itself an indicator of full transcriptional activity, especially in ESC) and methylated CpG abundance. Low CpG promoters (LCP) would be methylated regardless of their activity status, intermediate CpG promoter (ICP) activity would correlate inversely to the extent of methylation, and high CpG promoters (HCP) would have only weak or no methylation, even when inactive. Genome-wide high-resolution DNA methylation profiling across promoter regions was later carried out in mESC, superimposing chromatin state maps (histone modifications) on a given DNA methylation “background”; over 6100 genes that were hypermethylated over the promoter regions were annotated. DNA methylation in mESC occurs primarily in ICP and LCP, or in non-CpG island regions of HCP. Gene ontology analysis of the methylated genes showed that these genes encode sensory perception and signaling molecules, suggesting that methylated genes are involved mainly in late differentiation and signal transduction processes, and are not expressed in mESC; unmethylated promoters were associated with transcription, RNA and protein metabolic processes, cell survival and proliferation. This suggests that unmethylated promoters show reasonable correlation with genes that are active in ESC.

DNA methylation appears to be associated to specific histone marks.<sup>131</sup> For promoters occupied by histones methylated at H3K4 and/or H3K27, occupancy of promoter sequences by an activating histone mark (i.e., H3K4) correlates negatively with DNA methylation, while occupancy by a repressive histone mark (i.e., H3K27) correlates positively with DNA methylation. Promoters that lack both histone marks are highly methylated, and DNA methylation status is a good predictor of gene expression. During cell differentiation, hypermethylation can occur at CpG island promoters and at CpG-rich sequences outside promoter regions.<sup>131</sup> Remarkably, the authors claim that almost no demethylation is detected, and focus their attention mainly on HCP; they suggest that DNA methylation-mediated epigenetic repression increases globally during lineage specification. Many of the identified targets of differentiation-coupled *de novo* DNA methylation are stem cell- and germ-line-specific gene promoters.<sup>134</sup> One interpretation of this selectivity is that DNA methylation might stably repress the pluripotency program and prevent its aberrant re-activation and de-differentiation in physiological conditions. Experimental support for this model comes from a recent report showing that somatic cell reprogramming into iPS is greatly enhanced by treatment with the DNA methyltransferase inhibitor 5-azacytidine.<sup>135</sup> DNA methylation profiling of iPS compared to the primary fibroblasts from which they were derived clearly shows a large group of genes that are demethylated upon reprogramming, related mainly to pluripotency.<sup>136</sup> At the same time, many developmental genes are hypermethylated during reprogramming and correlate inversely with the presence of a bivalent mark.



Genetic and molecular data are thus compatible with a role for DNA methylation in the shut-down of pluripotency and, eventually, cell specification.

A powerful single-base mapping method was recently used to define the hESC methylome. Around one-quarter of all methylation is in a non-CG context (mCHG and mCHH, where H = A, C or T), a phenomenon almost unreported in animals, although there have been some studies. Methylation in non-CG contexts shows enrichment in gene bodies and depletion in protein-binding sites and enhancers; it disappears after induced hESC differentiation and is restored in iPS. Hundreds of differentially methylated regions were also identified proximal to genes involved in pluripotency and differentiation.<sup>137</sup>

In any case, the role of DNA methylation in lineage specification has not been elucidated exhaustively. Functional data demonstrate that treatment with the DNA demethylating drug 5-aza-2'-deoxycytidine greatly enhances cardiomyocyte differentiation from hESC or from the embryonic carcinoma cell line P19, as well as endothelial differentiation from mESC.<sup>138</sup> Genes involved in this differentiation effect have begun to be described; comparison of methylation profiles of hESC with adult tissue and in vitro-differentiated cells identified a group of genes (~12% of hESC hypermethylated genes) that are demethylated during differentiation.<sup>61</sup>

## EPIGENETICS OF HESC IMMUNOGENICITY

Overcoming their potential immunogenicity is one of the main challenges to stem cell use in regenerative medicine. hESC express very low levels of MHC class I molecules, whose antigen processing and presentation is necessary for T-cell recognition, and do not elicit immune responses in immunocompetent mice.<sup>139</sup> For these reasons, hESC are considered to have immune privilege properties.<sup>140,141</sup> It was recently shown that epigenetic mechanisms regulate low levels of MHC class I expression and lack of MHC class II in hESC.<sup>142</sup> Low MHC class I levels in hESC were specifically associated with reduced expression of TAP-1 and tapasin (TPN) as well as other antigen-processing machinery components (all of which are involved in peptide transport and loading) and with lack of  $\beta$ 2-microglobulin ( $\beta$ 2m) light chain; these factors limit expression of the MHC class I trimeric molecule at the cell surface. Epigenetic analysis in hESC and iPS showed that H3K9me3 repressed the *TPN* gene in undifferentiated cells, whilst HLA-B and  $\beta$ 2m acquired the activating H3K4me3 modification during ESC differentiation to embryoid bodies. Indeed, in vitro differentiation of these cells provokes re-activation of most of these promoters and MHC class I induction. Moreover, MHC class II expression is stably repressed in these cells through promoter hypermethylation of *HLA-DR* and *CIITA*, a transcription factor that specifically drives class II molecule expression.<sup>142</sup> These findings might help to refine techniques for modulating immunogenicity of hESC and their derived cells, thus avoiding their rejection after engraftment.

## CONCLUSION

Since its birth, epigenetics has been closely connected to development and differentiation. As a single cell generates a plethora of distinct specialized cells, all of which share the same genetic information, epigenetics must be involved in this process. Although our knowledge of development-related epigenetic mechanisms

has grown exponentially in recent decades, many questions remain to be answered. Here we have discussed several epigenetic mechanisms involved in the regulation of pluripotency and developmental genes in stem cells, and in their first differentiation steps. In addition, we showed that epigenetics can be involved in processes other than development and differentiation, such as antigen presentation and immunogenicity. It is hoped that knowledge of these mechanisms will lead to development of strategies to promote specific lineage differentiation, to induce tolerance, and to improve acceptance of stem cell allografts.

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## CHAPTER 17

# NEW TOOLS IN REGENERATIVE MEDICINE: Gene Therapy

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**Abstract:** Gene therapy aims to transfer genetic material into cells to provide them with new functions. A gene transfer agent has to be safe, capable of expressing the desired gene for a sustained period of time in a sufficiently large population of cells to produce a biological effect. Identifying a gene transfer tool that meets all of these criteria has proven to be a difficult objective. Viral and nonviral vectors, in vivo, ex vivo and in situ strategies co-exist at present, although ex vivo lenti- or retroviral vectors are presently the most popular.

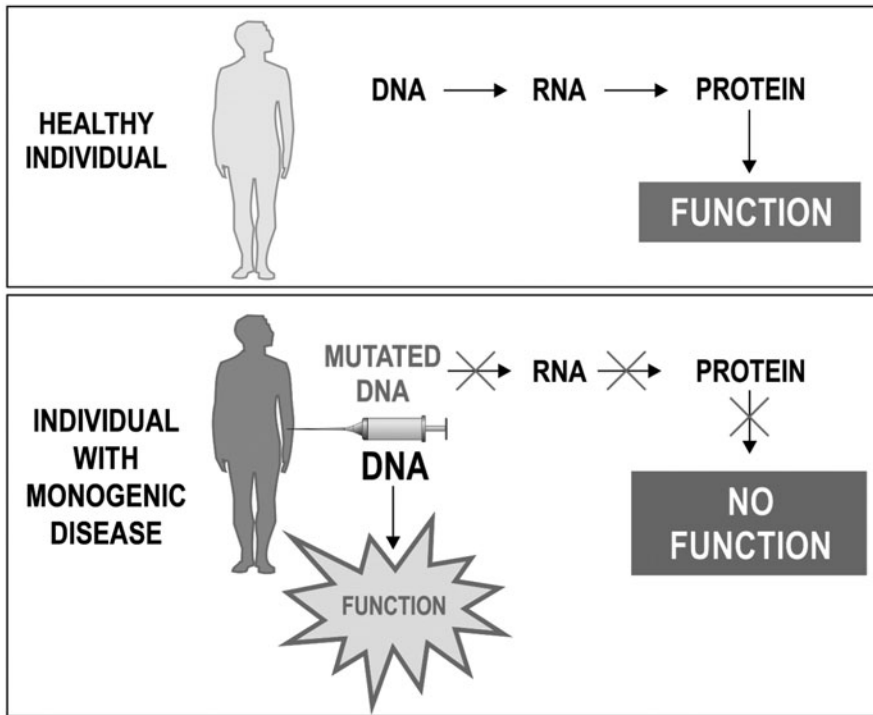
Natural stem cells (from embryonic, hematopoietic, mesenchymal, or adult tissues) or induced progenitor stem (iPS) cells can be modified by gene therapy for use in regenerative medicine. Among them, hematopoietic stem cells have shown clear clinical benefit, but iPS cells hold humongous potential with no ethical concerns.

## INTRODUCTION

Regenerative medicine aims to repair or replace lost or damaged cells<sup>1</sup>. This can be accomplished by stimulating natural regenerative mechanisms or by cell therapy and tissue engineering techniques of several cell types, including stem cells. Stem cell gene therapy widens their range of potential applications in regenerative medicine by providing new functions to complement or enhance their regenerative properties. Here, we will review basic gene therapy concepts, available vectors, delivery strategies, biosafety aspects, natural and induced stem cell as targets and current gene therapy clinical trials thereof. The technology is still in its infancy, but it has been used with some success and holds great therapeutic promise<sup>1</sup>.

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**Figure 1.** Schematic illustration of gene therapy. Insertion of genes into an individual's cell and biological tissues to treat disease, where deleterious mutant alleles are replaced with functional ones.

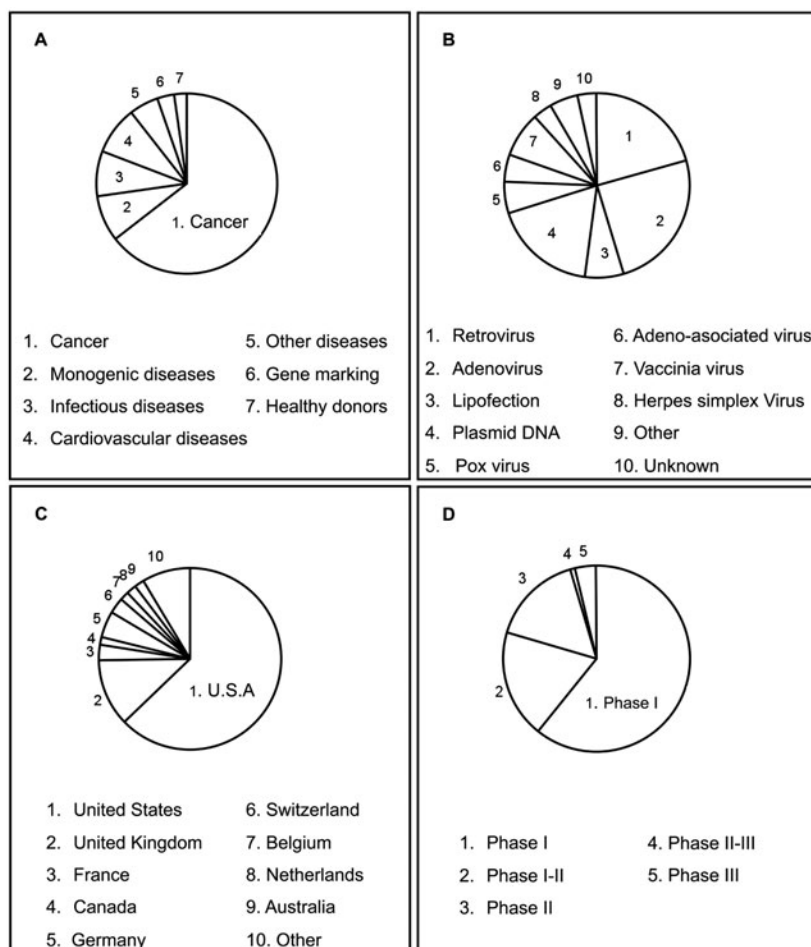
## CONCEPTS

### Gene Therapy

Gene therapy is the intentional delivery of genetic material to a patient with the aim of correcting a specific genetic defect with phenotypic consequences. More broadly speaking, it is a therapeutic technique by which a functional gene is inserted into the cells of a patient to correct a genetic abnormality or to provide those cells with a new function (Fig. 1). In any case, none of these definitions is sufficiently wide to cover the true range of current gene therapy, given the enormous dimensions and potential it has acquired recently.

This tool can be used to treat both hereditary diseases (e.g., cystic fibrosis and familial hypercholesterolaemia) as well as acquired diseases (e.g., cancer and vascular diseases). At the start, the aim of gene therapy was to correct genetic deficiencies by inserting into abnormal cells normal genes which would be able to take on the functions that the faulty genes were not performing (e.g., X-linked severe combined immunodeficiency). Later, a new type of gene therapy was developed in which the gene inserted was able to give the patient's cells new properties (e.g., anti-tumour therapies through "suicide genes"). Both types continue to be used today, although most of the protocols used currently belong to this latter approach and offer new strategies to fight cancer (Fig. 2A). Overall, very few gene therapy protocols have proved to be both safe and efficient and thus reached





**Figure 2.** This figure shows information about 1644 protocols of gene therapy in humans over the world. A) Most protocols are designed to treat cancer. B) Retroviral and adenoviral vectors have been the most commonly used vectors in gene therapy protocols in humans. Non viral vector have been used in only 25 % of the cases. C) Most of the gene therapy protocols have been developed by U.S. nowadays. D) Most protocols, are in Phase I (biosecurity). Only a small percentage are in Phase II or III, to test the efficacy of therapy. Reprinted from Gene Therapy Clinical Trial Worldwide (Journal of Gene Medicine), <http://www.abedia.com/wiley>, with permission of Wiley Publishers.

commercialisation (Fig. 2D). One example is the compound “Fomiversen”, approved by the FDA, which is an antisense oligonucleotide to treat cytomegalovirus (CMV)-induced retinitis in immunosuppressed patients.

Gene therapy can be classified under two categories, according to its possible targets: Somatic gene therapy (somatic cells) and germinal gene therapy (egg cells, sperm and their corresponding precursors). The fact that genetic alterations produced in germinal cells, as opposed to somatic cells, can be passed on to descendents entails a whole set of bioethical and social implications which could be the subject of another entire chapter.



The future of gene therapy depends on the resolution of various methodological obstacles in order that safe and more efficient gene transfer systems are developed. Accomplishing this, while respecting bioethical principles, would turn gene therapy into a valuable alternative to conventional treatments for a great number of diseases.<sup>2</sup>

### Transgene

A transgene is defined as a “gene which has been transferred into the genome of another organism”. The first attempts to carry out gene therapy in higher organisms were performed on mice. In those experiments, foreign (exogenous) recombinant DNA was called a transgene and recipient animals were termed transgenic.

At the beginning, the transgene used to be injected into early embryos so that it would be incorporated into the chromosomes of certain embryo cells, where it would remain and proliferate until those cells became mature cells. Some attempts were also made at integration into the germinal line, but real progress was not seen until gene cloning techniques were developed. The improved technique consisted of injecting cloned genes into an egg cell which had been fertilized with two pronuclei, one male and one female, which would become the nucleus of the single cell embryo. Later, the modified single-cell embryo would be transferred to the uterus of a female where it would resume its development until birth. This technique had severe limitations since fewer than 30% of zygotes survived and even fewer embryos reached term with the transgene integrated (2-30%). Those first trials raised concerns about where and when the expression of that genetic material took place. The main problems are the following:

- Position effects due to the site of the integration being undetermined
- Multiple integration, either tandem integration or not
- Variable expression, called variegation when it happens within lines
- Methylation and lack of expression
- Mosaicism

Currently gene therapy can draw on different techniques:

- Gene insertion (nonhomologous recombination), where a normal version of the altered gene is inserted into target cells, without actually modifying the gene itself.
- Genetic surgery (homologous recombination), where the faulty gene is replaced by a version of the gene which functions normally. This is what zinc-finger nuclease fusion proteins can achieve. They are designed to recognise a certain gene carrying the zinc-finger domain. When they do so, the nuclease domain cuts the target sequence and stimulates homologous recombination with a normal version of the relevant sequence also carried by the fusion protein.<sup>3</sup>
- Genetic modification, a technique in which the faulty gene is not replaced, but rather repaired by directed mutagenesis. This can be achieved, for example, by using bi-functional triplex forming oligonucleotides (TFOs), which contain a repair domain and an effector domain. The latter not only directs the complex to the region of the genome where there is a mutation, but also acts as an inducer of homologous recombination. Thus the mutated DNA is replaced by the wild-type sequence without any insertion difficulties.<sup>4</sup>

To date, most protocols have used gene insertion despite the risks involved. However, genetic surgery and modification also have various limitations, such as their low efficiency the uncertainty of whether it is just the targeted nucleotide that is repaired (there may be several similar sequences belonging to different genes which would be altered with unforeseeable consequences) and the fact that the new protein may present a new epitope (or several new epitopes), which could then induce an autoimmune response, something that can also occur in the case of insertion.

Nonetheless, genetic surgery and modification offer plenty of advantages with respect to insertion. Specifically, the protocols used for genetic surgery cannot affect the switching on and off of genes (e.g., oncogenes); likewise, the genome is not lengthened, antigenicity should be lower than in gene insertion because the change is likely to be smaller and particularly because the contexts of gene regulation and expression are not altered.<sup>5</sup>

## VECTORS

To obtain a particular biological effect using gene therapy, the relevant gene sequence must be effectively inserted into the target cell and then be expressed. Achieving these goals requires having the appropriate carriers and, also, the appropriate promoters to ensure the maximum expression of the genetic material introduced. Vectors are systems that contribute to the process of introducing the foreign genes into the cell, thereby facilitating their entrance and bioavailability so that it can function correctly.

The large number of potential target cells in gene therapy justifies work searching among the different types of vectors according to their strengths and to the characteristics of the pathology to be treated. The attainment of a universal vector for generic use is therefore a utopia, given that different diseases tend to have opposing requirements. However, there are certain desirable features that any such tool should have and which may be summarised by the following top ten requirements:

1. Reproducible and stable
2. Fully characterisable
3. Innocuous, with low immunogenicity and no side effects
4. Lineage-specific (acting specifically on cells which are affected)
5. Effective with both dividing and quiescent cells
6. Able to be produced at high titres (higher than  $10^8$  particles/ml)
7. Allow the regulation of the expression of the therapeutic gene
8. Allow the specific integration of the therapeutic gene (homologous recombination)
9. Able to carry the therapeutic gene regardless of its size
10. Easily produced and stored, at an affordable cost

Indeed, the limiting factor for gene therapy is currently this area of methodology. A great number of vectors have been developed to date<sup>6</sup> but so far none of them has reached the required quality to turn gene therapy into an actual, real and competitive alternative to traditional therapies.

There are two main types of vectors: Viral and nonviral. All vectors obtained from a virus belong to the first group. This tool tries to benefit from the evolutionary strengths of these organisms as natural vectors and eliminate their pathological characteristics, while enhancing their ability to transport genetic material. Nonviral vectors, on the

other hand, have a different approach, that of synthesis instead of modification. This type of vector is based on building a totally artificial system for transporting genetic material using known molecular structures. There are pros and cons to both types of vectors which condition their applicability to the various different existing gene therapy protocols.

### **Viral Vectors**

Currently the most effective vectors (Table 1), these were the first to be employed in clinical trials, in the early nineteen-nineties, and are still the most widely used option (Fig. 2B). Their production for therapeutic purposes involves the following steps. First, their ability to propagate must be destroyed by eliminating the genes responsible for their replication. Then, in order to obtain viral particles, other cell lines, known as “packing cell lines”, are used to carry part of the viral genome and allow the completion of the viral replication cycle. These lines may also code for proteins from other viruses, which contribute to the viral envelope (e.g., VSV-G from the vesicular stomatitis virus). Hence, the tropism of the viral particle towards a certain cell lineage may be modified or enhanced (pseudotyping). The next step concerns the creation of enough space to be able to introduce the relevant genetic material including the therapeutic gene or genes, plus the required control and safety elements. This gene substitution process must be closely monitored so that any sequences that are potentially harmful to the target cell are eliminated while the infective ability of the vector is maintained.<sup>7</sup>

The main advantages of viral vectors are, on the one hand, their high transfer efficiency and, on the other, the possibility that some of them integrate the transgene into the genome of the host cell. The drawbacks of this type of systems stem from the transfer and expression of viral sequences in an uncontrolled manner. Such is the case when a native virus is accidentally transferred or when a second pathogenic virus or an oncogene is activated due to nonspecific integration. Besides these issues, there are other limitations that should not be forgotten, such as the size of the genetic material to be incorporated (given that it is constrained by the viral packaging process) and the immune response of the recipient, which can jeopardize genetically modified cells due to a change in their immunogenicity.

### **Nonviral Vectors**

Nonviral or physico-chemical methods of gene transfer were the first to be developed (Table 2). In these methods,<sup>8</sup> foreign DNA is integrated into a plasmid, which is a DNA molecule which can be maintained episomically, that is to say, in a stable way independent of the genome of the host cell. Advantages offered by this type of methods include:

- Greater stability and biosafety
- Easy to manipulate, which allows their industrial-scale production
- No restrictions with regards to the DNA size they are able to transfer
- Low toxicity and a lower immunogenicity than viral vectors
- Some of them exhibit high cell specificity

Their main drawback is that, in most cases, they have lower transduction efficiency than their viral analogues.

Table 1. Most commonly used viral vectors

Original Virus	Nucleic acid	Maximum Transport Capacity	Immunogenicity	Integration of Transgene	Stability/ Specificity	Target in Cycle?	Efficiency
<i>Retrovirus</i>	Double-stranded RNA	7-8 kb	Low	Yes (random)	Low/Low	Yes (except lentivirus)	High
<i>Adeno-associated virus</i>	Single-stranded DNA	4.5 kb	Low	Yes (specific)	High/Low	No	High
<i>Herpesvirus</i>	Double-stranded DNA	Up to 30 kb	Medium	No	High/Low	No (except some gamma-herpesvirus)	High
<i>Adenovirus</i>	Double-stranded DNA	Up to 36 kb ("gutless*")	High	No	High/Low	No	High
<i>Vaccinia virus</i>	Double-stranded DNA	25 kb	High	No	Low/Low	No	High

\* "Gutless": 3rd generation adenoviral vector with high transport capacity due to the elimination of all viral coding regions. No cellular immune response are generated, but unlike their predecessors they require "helper" adenovirus to provide all viral proteins.

**Table 2.** Most commonly used non-viral vectors

Description	Nucleic Acid	Maximum Transport Capacity	Immunogenicity	Integration of Transgen	Stability/Specificity	Target in Cycle?	Efficiency
<i>Physical methods (microinjection, electroporation, microparticle bombardment, etc.)</i>	Any	Ilimited	Very low	No <sup>&amp;</sup>	High/High (local application)	No*	High, but very localized
<i>DNA-protein conjugates (Peptiplexes)</i>	Any	Ilimited	Low	No <sup>&amp;</sup>	High/High*	No*	Medium
<i>Cationics liposomes (Lipoplexes)</i>	Any	Ilimited	Very low	No <sup>&amp;</sup>	High/Low*	No*	Medium
<i>DNA-polycations conjugates (Poliplexes)</i>	Any	Ilimited	Low	No <sup>&amp;</sup>	High/High*	No*	Medium
<i>DNA-HVJ<sup>ç</sup> virus conjugates<sup>ç</sup> (Virosomes)</i>	Any	Ilimited	Very low	No <sup>&amp;</sup>	High/High*	No*	Medium

\* In some cases.

ç Hemagglutinating virus of Japan or Sendai virus (HVJ): DNA-protein complexes are encapsulated in classic liposomes, after they are merged with the inactivated virus by ultraviolet light.

& The integration ability into host genome is in process of development in these systems. An example is the use of transposons such as “Sleepy Beauty”, able to insert transgenes into mammalian chromosomes.

The development of vectors is one of the main technical challenges for gene therapy today and has been the greatest limitation on satisfactory outcomes achieved so far. So much so that some leading researchers, such as T. Friedmann (University of California, San Diego, USA), professed that for the future strategies must change substantially: “Almost for certain the tools of the future are not going to be the prototypes which are currently being tested in the laboratories. And there will not be an ideal technique for each disease, rather there will be many options”. Accordingly it can be stated that safety in the development and use of these tools together with the improvement of their efficiency are the key factors for the future of these protocols.

## **THERAPEUTIC STRATEGIES**

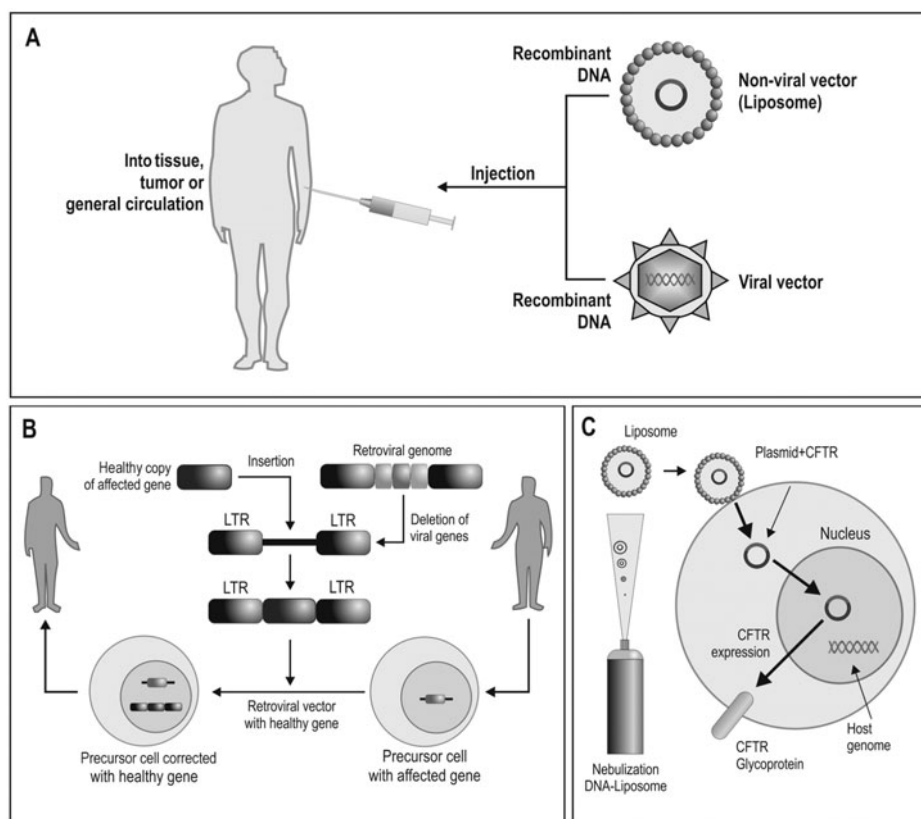
There are three different approaches to gene transfer protocols at the present time which, combined with the different types of vectors, offer a wide range of possibilities. As mentioned at the beginning of this chapter, this allows us to choose the most appropriate option according to the characteristics of the pathology to be treated.

### **In Vivo Gene Therapy**

This includes techniques in which therapeutic genetic material is delivered using appropriate vectors through the bloodstream to the target cells, without subjecting them to any kind of manipulation outside the patient (Fig. 3A). This strategy is advantageous due to its methodological simplicity, since selective delivery of the gene to the chosen organ or tissue is achieved without resorting to traumatic procedures or surgery. However, this technique is not free of disadvantages. There is less tight control over the gene transfer process than with *in vitro* techniques; overall efficiency (number of cells modified) is also lower, since they cannot be selected and expanded; and, finally, it is not easy to attain a high degree of specificity as far as the target cell is concerned, because target-specific vectors are required. Another possibility is to modify, for example, skin<sup>9</sup> or liver cells with the aim synthesising and secreting into the bloodstream proteins that the patient lacks and that are normally produced by other cell types. In this way, therapeutic material is constantly bioavailable, without requiring additional drugs.

### **Ex Vivo Gene Therapy**

This includes protocols in which cells to be treated are previously extracted from the patient, isolated and grown in culture before subjecting them to the gene transfer process (Fig. 3B). Once the protocol has been carried out, cells which have been successfully transduced are expanded in culture to then re-introduced into the patient. These techniques offer various advantages including that they provide a high level of control over the transfer process, show a high degree of transduction efficiency and allow selection of the cell-type to be treated. The main disadvantages stem from the greater complexity and cost of the protocols as well as from problems associated with cell cultures (contamination, difficulties for growing certain cell lineages *ex vivo*, etc.). Severe combined immunodeficiency may be cited as an example of the application of this strategy.



**Figure 3.** Schematic illustration of the different strategies used in gene therapy. A) In vivo: the therapeutic genetic material is carried by the relevant vector and introduced into the body through the bloodstream. B) Ex vivo: the cells that will be treated are manipulated outside the body, and after a complex process of selection and expression, they are reintroduced to complete their missions. C) In situ: the genetic material is transferred directly to the target organ, where therapy is required.

### In Situ Gene Therapy

This includes techniques whereby genetic modification of the cells is achieved by introducing therapeutic DNA directly into the affected organ (Fig. 3C). Its greatest advantage lies in its high specificity given the local delivery straight into the organ. A case in point is the mouse model of Duchenne's muscular dystrophy.<sup>10</sup> Recombinant plasmids were built from normal human genes and they were directly injected into the skeletal and cardiac muscle of mutant mice. Expression of transferred genes proved to be stable over a period of months and the plasmid remained circular and independent of the main body of genetic material in the nonreplicative context of the muscle fibre. Unfortunately, the level of expression turned out to be too low to have a therapeutic effect (10 to 50 times lower than desirable). This strategy is therefore most appropriate for the treatment of disorders such as cystic fibrosis and muscular dystrophy and for the suppression of tumours by cell "suicide".



## BIOSAFETY ASPECTS

Stem cell gene therapy is considered in the realm of “advanced therapy medicinal products” as defined by the European Council in its three main types: (1) gene therapy, (2) somatic cell therapy, and (3) tissue engineered products. Therefore, production of modified stem cells that will go into patients, even for clinical trials, must comply with Good Manufacturing Practice (GMP) for medicinal products.

Typical regulatory concerns for stem cells are product safety, cell characterization, and control of their manufacturing process.<sup>11</sup> With regard to safety, cell donors must be carefully screened and the cellular product, once expanded in the GMP production facilities through master and working cell banks, must be checked by standardized tests for viability, sterility, adventitious agents, genetic stability/tumorigenicity, pyrogenicity, mycoplasma infection, etc. Stem cell products will also have to be defined for identity, purity, potency, stability, and viability.

Before administration into humans, both the biological activity and toxicity of stem cells must be tested in a relevant animal model under Good Laboratory Practice (GLP) conditions.

Despite all this hurdles, the gap between bench and bed is rapidly closing.<sup>12</sup>

## APPLICATIONS FOR REGENERATIVE MEDICINE USING STEM CELLS

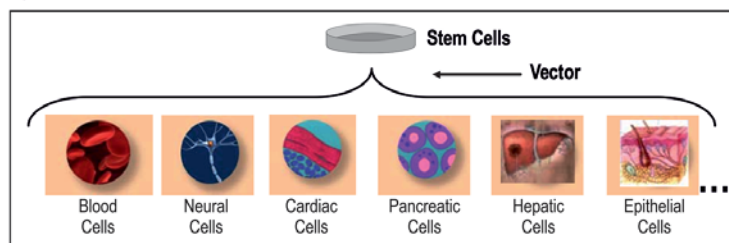
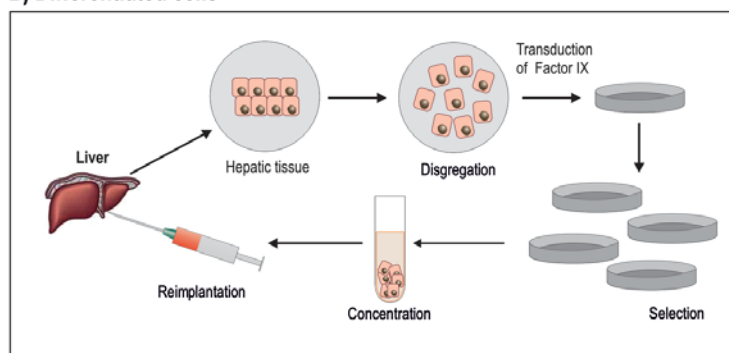
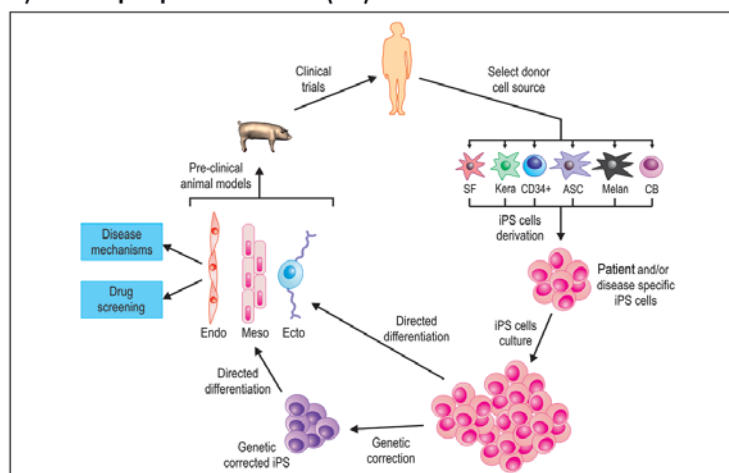
Regenerative medicine seeks to repair or substitute damaged cells, tissues and organs so that their functions are restored (Fig 4). This can be achieved either by stimulating natural regenerative mechanisms or by cell therapy and tissue engineering techniques, particularly—but not only—using stem cells. Within the second of these approaches, modification by gene therapy of the cells to be used in regenerative medicine widens the range of potential applications, as it provides new functions to complement or improve the cells’ regenerative properties, and this corresponds to the subject of this chapter.

Some of these applications have already become a reality. This is the case of gene therapy of hematopoietic stem cells from patients with congenital immunodeficiency disorders,<sup>13</sup> which has proved its ability to emulate, through autologous transplantation of genetically modified cells, what compatible allogeneic bone marrow transplantation had achieved many years earlier.<sup>14</sup>

We shall now consider those applications according to whether the target cells (somatic cells) are natural or induced stem cells, with emphasis on outcomes in humans.

### Natural Stem Cells

Embryonic stem cells (ESC) have the most regenerative potential, given their great capacity for self-renovation and differentiation to any tissue, under appropriate culture conditions. Even if we are still far from their clinical application, a great deal of research has been carried out on them in recent years, including the possibility of using them as gene therapy targets.<sup>15</sup> Ethical issues related to human ESC have led to the search for sources of non-embryonic stem cell with equivalent properties. Attempts have included the reprogramming of differentiated cells into induced pluripotent stem (iPS) cells<sup>16</sup> and the isolation of adult tissue stem cells (TSC).<sup>17</sup> While bone marrow stem cells are the

**A) Stem Cells****B) Differentiated cells****C) Induced pluripotent stem cells (iPS)**

**Figure 4.** Applications of gene therapy in regenerative medicine depending on the properties of the target cell. A) Stem Cells (see “Natural stem cells”, ESC, HSC, MSC, TSC) are those with the highest regenerative potential, which may lead, under appropriate culture conditions, to any kind of tissue. Gene therapy in these cells has therefore a great potential. B) Differentiated cells: the figure shows one of the gene therapy strategies used with hepatocytes in the treatment of haemophilia B (lack of factor IX, 33), but there are protocols in other differentiated cells such as lymphocytes, neurons or muscle cells. C) iPS cells. There remain significant hurdles to overcome in each step, from iPS cell derivation to pre-clinical trials, before iPS cell-based clinical applications can become a reality. SF, skin fibroblasts; Kera, keratinocytes; CD34<sup>+</sup>, CD34<sup>+</sup> cells from peripheral blood; ASC, adipose stem cell; CB, cord blood cell; Endo, endoderm; Meso, mesoderm; Ecto, ectoderm. Reprinted with permission from: Sun N et al. *Cell Cycle*. 2010; 9(5):880-5.<sup>32</sup>

most widely known, many other tissues have been explored with successful outcomes, such as the umbilical cord, adipose tissue<sup>18</sup> and skin tissue.

Bone marrow contains two types of TSC, hematopoietic stem cells (HSC) and nonhematopoietic or mesenchymal stem cells (MSC). The former are more abundant and have proved their ability to differentiate into myeloid and lymphoid lineages, while the latter can differentiate into other lineages (adipocytes, myocytes, fibroblasts and stroma).<sup>19</sup> The majority of gene therapy applications have been tested using HSC, but results from MSC are now beginning to emerge.<sup>20</sup>

### *Embryonic Stem Cells*

Embryonic stem cells have been induced to differentiate into cells with hepatocyte properties so in time they will be used as a source for this cell lineage. A particular advantage of these stem cells is that they can be used for gene manipulation, including gene therapy for congenital liver defects<sup>21</sup> and nuclear transplantation,<sup>22</sup> which would make it possible to produce ESCs compatible for each patient.

### *Hematopoietic Stem Cells*

Hematopoietic stem cells are capable of repopulating the whole hematopoietic system from just a few precursors. This regenerative property has been responsible for some spectacular applications, such as bone marrow transplantation, both autologous (to restore haematopoiesis after myeloablative cancer treatments) and allogeneic transplantation (for congenital immunodeficiencies—IDs). What has been learnt to date has eased the way to gene supplementation of autologous HSC for these IDs, which, thanks to an additional functional gene, has restored lost functions for their progeny. There is at least one case in which they have proved to be clinically effective beyond doubt.<sup>13</sup> The two most common severe combined immunodeficiencies (SCIDs), characterised by a drastic reduction of T-lymphocytes, have been successfully treated: Both the X-linked ID (due to mutations in the  $\gamma$  chain present in many cytokine receptors) and non-X-linked one (due to mutations in the adenosine deaminase enzyme—ADA).

Two studies with a total of 20 patients suffering from the first of these diseases were carried out using an ex vivo retroviral system on CD34<sup>+</sup> HSC. Treated cells were re-introduced into the patients, who quickly recovered normal levels of T lymphocytes, all of the progeny carrying the transgene. Transduced HSC (<2%) were still present several years later, which indicates that the selective advantage of corrected progeny was responsible for the great expansion of the healthy T-cell lineage. Both studies showed a severe side effect, acute lymphoblastic leukaemia, in total of five patients, as a result of the vector-associated insertional oncogenesis. In four of these cases, the pathology was caused by the activation of the LMO-2 proto-oncogene due to the viral LTR enhancer.

In another two studies, thirteen patients with ADA deficiency were successfully treated, at least in part thanks to the absence or elimination of the substitution treatment with ADA-PEG (to increase the selective advantage of the corrected lymphocytes) and to the partial myeloablation (to facilitate grafting of treated HSC). For other IDs treated using a similar strategy, such as chronic granulomatous disease, the lack of selective advantage only yielded temporary results.<sup>23</sup>

In AIDS, an acquired ID, anti-HIV gene therapy strategies have been tested *ex vivo* on HSC and self-transplantation then attempted, but without partial myeloablation. In this case, a few months after the procedure hardly any transgenic cells remain in the bloodstream, although there is evidence of some selective advantage in modified HIV resistant mature lymphocytes.<sup>24</sup>

### *Mesenchymal Stem Cells*

Mesenchymal stem cells can be isolated from bone marrow as well as from blood, the placenta, dental pulp, adipose tissue and the umbilical cord.<sup>25</sup> MSCs demonstrate two interesting properties for the field of HSC transplantation: (1) their ability to support haematopoiesis improves grafting and (2) their immunomodulatory effect is an asset to treat graft-versus-host disease.<sup>26</sup> However, it is their pluripotent nature<sup>19</sup> and their ability to nest in metastatic tumours and in damaged tissues that have broadened their possible applications in the field of regenerative medicine and gene therapy against cancer and other metabolic diseases. For instance, it has been proved in animal models that human MSC modified to express IFN- $\gamma$  are incorporated into human tumour tissue (specifically melanoma and breast cancer) and are able to inhibit its growth *in vivo*.<sup>27</sup> Ectopic expression of adhesion molecules in murine MSCs allows their migratory properties to be modified in favour of the tissue of interest (for example, expression of  $\alpha 4$  integrin increases their retention in bone).<sup>28</sup>

### *Other Tissue Stem Cells*

Various pseudotyped lentiviral vectors have been used to express  $\beta$ -galactosidase in human skin which has been transplanted into SCID mice,<sup>29</sup> demonstrating transduction of epidermal stem cells. A very positive outcome has been reported for a Phase I clinical trial on a single patient suffering from epidermolysis bullosa.<sup>30</sup> The procedure consisted of transplantation of autologous epidermal stem cells which had been corrected using a retroviral vector. This vector encoded the laminin-5  $\beta_3$  chain, the gene for which is mutated in this rare recessive blistering disease. This trial provides proof of principle for the procedure in this and other skin diseases, although safety with self-inactivating (SIN) vectors must be improved and the issue of the immune response to neoantigens must be addressed (the treated patient had low levels of the mutant protein, which may explain why no response was induced against the normal transgenic  $\beta_3$  chain).

### **Induced Pluripotent Stem Cells**

The ectopic expression of one to four transcription factors can reprogram human somatic cells into a pluripotent state termed iPS.<sup>16</sup> In essence, therefore, iPS cells are the product of gene therapy to endow a somatic cell with regenerative properties. iPS cells are very similar, but not identical, to embryonic stem cells (ESC), which can differentiate into every somatic cell-type of the human body and show unlimited replication. To their credit, iPS cells can bypass the ethical concerns associated to ESC derivation and potentially their allogeneic rejection. Therefore, they may be a better source, and they are now a leading candidate, of patient-specific and disease-specific adult cells for future regenerative medicine applications.<sup>31</sup> iPS cell-based therapies

also need to be thoroughly evaluated in the preclinical animal models before they can be applied to human subjects.

However, a number of obstacles need to be cleared before patient-specific iPS cells can advance into the clinic.<sup>32</sup> Which somatic cell should be used? Naturally it is best if the cells are easy to access, culture and reprogram. Most studies use skin fibroblasts, although their reprogramming efficiency is relatively low (<0.01%) and slow (3-4 weeks). Other options include neural, adipose, peripheral blood or cord blood stem cells, as well as keratinocytes or melanocytes. Each has its own pros and cons and it will probably be a patient-specific decision. Which gene therapy vectors should be used? Lenti- or retroviral vectors are the most popular, but transposons, proteins or DNA have been used in an attempt to generate virus-free, transgene-free autologous iPS in the future. What is an iPS? Standard markers are still lacking, and reprogramming experiments generate multiple heterogeneous cell lines which may not always show sufficient safety and pluripotency. What are the best culture conditions for iPS? Currently iPS, as ESC, are grown on mouse embryonic fibroblasts (MEFs) to ensure their proliferation in an undifferentiated state. But contamination with animal pathogens can occur, and feeder-free culture methods are being developed. Which is the best preclinical model for iPS? The teratoma potential of undifferentiated iPS as well as the insertional oncogenesis of current vectors on their differentiated progeny need to be rigorously tested in small (mouse, rat) and large (monkey, pig) animal models.

### Ongoing Clinical Trials

Searching for stem cell gene therapy in <http://clinicaltrials.gov/renderers> 156 clinical trials, which once revised for relevance are reduced to 26 (Table 3A). As explained above, most are done with hematopoietic stem cells. For iPS only 6 trials are relevant (Table 3B), and none involve using the iPS in vivo. Nevertheless, stem cell gene therapy applications, including iPS cells, are here to stay. With further improvements they have the potential to revolutionize regenerative medicine.

### CONCLUSION

Stem cell gene therapy is here to stay. With further improvements it will revolutionize regenerative medicine.

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**Table 3.** Summary table searching for stem cell gene therapy in <http://clinicaltrials.gov/>

Rank	Status	Study
A) Searching for stem cell gene therapy in <a href="http://clinicaltrials.gov/">http://clinicaltrials.gov/</a> , which once revised for relevance are reduced to 26		
1	Active, not recruiting	Stem cell gene therapy to treat X-Linked Severe Combined Immunodeficiency (XSCID)  Condition: Severe combined immunodeficiency Intervention: Drug: gene-transduced autologous CD34 <sup>+</sup> stem cells
2	Active, not recruiting	Gene therapy-treated stem cells in treating patients undergoing stem cell transplant for intermediate-grade or high-grade AIDS-related lymphoma  Condition: Lymphoma Interventions: Biological: filgrastim; biological: gene therapy; drug: carmustine; drug: cyclophosphamide; drug: etoposide
3	Completed	The effect of mobilized stem cell by G-CSF and VEGF gene therapy in patients with stable severe angina pectoris  Condition: Ischemic heart disease Intervention: Genetic: VEGF-A165 plasmid
5	Completed	ADA gene transfer into hematopoietic stem/progenitor cells for the treatment of ADA-SCID  Condition: Severe combined immunodeficiency Intervention: Genetic: gene transduced CD34 <sup>+</sup> cells
6	Active, not recruiting	Combination chemotherapy plus gene therapy in treating patients with CNS tumors  Conditions: Bone marrow suppression; brain and central nervous system tumors; drug/agent toxicity by tissue/organ Interventions: Procedure: filgrastim; biological: gene therapy; drug: lomustine; drug: procarbazine hydrochloride; drug: vincristine sulfate

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Table 3. Continued

Rank	Status	Study
7	Recruiting	Gene therapy for chronic granulomatous disease Condition: Chronic granulomatous disease Intervention: Drug: phagocyte oxidase subunit transduced CD34 hematopoietic stem cells
8	Completed	Gene therapy in HIV-positive patients with non-Hodgkin's lymphoma Conditions: Lymphoma, non-Hodgkin; HIV infections Intervention: Drug: peripheral blood stem cells
9	Active, not recruiting	Gene therapy for chronic granulomatous disease in Korea  Condition: Chronic granulomatous disease Intervention: Drug: autologous hematopoietic stem cells transduced with MT-gp91 retroviral vector
10	Completed	Gene therapy for ADA-SCID Condition: Severe combined immunodeficiency syndrome Intervention: Genetic: gene transduced PBL and/or gene transduced HSC
12	Completed	Gene therapy for the treatment of Fanconi's anemia Type C Conditions: Fanconi's anemia; pancytopenia Intervention: Drug: transduced CD34 <sup>+</sup> cells
13	Terminated	Gene therapy and chemotherapy in treating patients with advanced solid tumors or non-Hodgkin's lymphoma Conditions: Brain and central nervous system tumors; lymphoma; unspecified adult solid tumor, protocol specific Interventions: Biological: filgrastim; biological: sargramostim; biological: therapeutic autologous lymphocytes; drug: O6-benzylguanine

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**Table 3.** Continued

Rank	Status	Study
14	Active, not recruiting	Gene therapy, chemotherapy, and peripheral stem cell transplantation in treating patients with HIV-related non-Hodgkin's lymphoma Condition: Lymphoma Interventions: Biological: RevM10 gene; Biological: RevM10/polAS gene; Procedure: In vitro-treated peripheral blood stem cell transplantation
15	Recruiting	MND-ADA transduction of CD34 <sup>+</sup> cells from children with Adenosine Deaminase (ADA)-deficient Severe Combined Immunodeficiency (SCID) Condition: Severe combined immunodeficiency Intervention: Biological: ADA gene transfer
16	Completed	Antimetabolite induction, high-dose alkylating agent consolidation and retroviral transduction of the MDR1 gene into peripheral blood progenitor cells Conditions: Breast neoplasms; Neoplasm metastasis Intervention: Genetic: Peripheral blood progenitor cells carrying MDR1
19	Withdrawn	Stem cell mobilization and VEGF gene transfer for heart failure Condition: Ischemic Congestive Heart Failure (CHF) Intervention: Genetic: Vascular Endothelial Growth Factor (VEGF1)
20	Completed	Stem cell (modified bone marrow) transplantation in HIV-Infected patients with blood cancer Conditions: Hematologic neoplasm; HIV Infection Intervention: Drug: GCSF mobilized allogeneic PBSC cultured w/Cytokines; Transduced w/RV
21	Recruiting	Pulmonary hypertension: Assessment of cell therapy Condition: Hypertension, Pulmonary Intervention: Biological: Transfected cells will be delivered via a PA line

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Table 3. Continued

Rank	Status	Study
23	Completed	Trial of an anti-HIV-1 gene transfer product Condition: HIV Infections Intervention: Genetic: OZ1 (anti-HIV-1 gene)
24	Recruiting	Gene transfer therapy for Severe Combined Immunodeficiency Disease (SCID) due to Adenosine Deaminase Deficiency (ADA) Condition: Severe combined immunodeficiency syndrome Intervention: Drug: CD34+ cells transduced with ADA retrovir
25	Completed	Gene therapy for patients with leukocyte adherence deficiency (follow-up of Phase 1 trial) Condition: Leukocyte adhesion deficiency syndrome Intervention:
27	Recruiting	Gene transfer for Severe Combined Immunodeficiency, X-linked (SCID-X1) using a Self-inactivating (SIN) gammaretroviral vector Condition: Severe combined immunodeficiencies Intervention: Biological: Retrovirus-mediated gene transfer
28	Active, not recruiting	Gene therapy for chronic granulomatous disease Condition: Granulomatous disease, chronic Intervention: Drug: Retroviral SF71-gp91phox transduced CD34+ cells

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**Table 3.** Continued

Rank	Status	Study
52	Recruiting	High-dose chemotherapy with transplantation of gene-modified stem cells for high-risk AIDS-related lymphoma Conditions: AIDS-related lymphoma; HIV infections Intervention: Procedure: PBSC-M87o, gene (M87o)-modified, CD34 <sup>+</sup> peripheral blood progenitor cells (PBSC)
57	Active, not recruiting	Phase I pilot study of CD34 enriched, Fanconi's anemia complementation group C gene transduced autologous peripheral blood stem cell transplantation ith Fanconi's anemia Condition: Fanconi's anemia Interventions: Drug: filgrastim; genetic: autologous stem cells transduced with FACC retroviral vector; procedure: autologous stem cell transplantation
97	Completed	Phase I study of retrovirally mediated transfer of the human glucocerebrosidase gene into peripheral blood stem cells for autologous transplantation Condition: Gaucher's disease Intervention: Genetic: human glucocerebrosidase gene into autologous peripheral blood stem cells
106	Recruiting	Patient specific induced pluripotency stem cells (PSiPS) Conditions: Hepatic disorders; eye disorders Intervention: Procedure: biopsy

*continued on next page*

Table 3. Continued

Rank	Status	Study
B) Relevant iPS trials		
1	Active, not recruiting	Derivation of induced pluripotent stem cells from an existing collection of human somatic cells  Condition: Amyotrophic lateral sclerosis Intervention:  Use of existing fibroblast cells to convert to induced pluripotent stem cells  Conditions: Pregnant, healthy females; healthy male newborns Intervention:  Development of iPS from donated somatic cells of patients with neurological diseases  Condition: Neurodegenerative disorders Intervention:
4	Recruiting	Skin and blood research samples from healthy volunteers and sickle cell anemia patients  Condition: Anemia, sickle cell Intervention: Procedure: skin and blood samples
5	Recruiting	Patient specific induced pluripotency stem cells (PSiPS)  Conditions: Hepatic disorders; eye disorders Intervention: Procedure: biopsy
7	Recruiting	Establishing fibroblast-derived cell lines from skin biopsies of patients with immunodeficiency or immunodysregulation disorderslymphohistiocytosis, hemophagocytic; common variable immunodeficiency;  Conditions: Severe combined immunodeficiency Intervention:

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## THERAPEUTIC CLONING AND CELLULAR REPROGRAMMING

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**Abstract:** Embryonic stem cells are capable of differentiating into any cell-type present in an adult organism, and constitute a renewable source of tissue for regenerative therapies. The transplant of allogenic stem cells is challenging due to the risk of immune rejection. Nevertheless, somatic cell reprogramming techniques allow the generation of isogenic embryonic stem cells, genetically identical to the patient. In this chapter we will discuss the cellular reprogramming techniques in the context of regenerative therapy and the biological and technical barriers that they will need to overcome before clinical use.

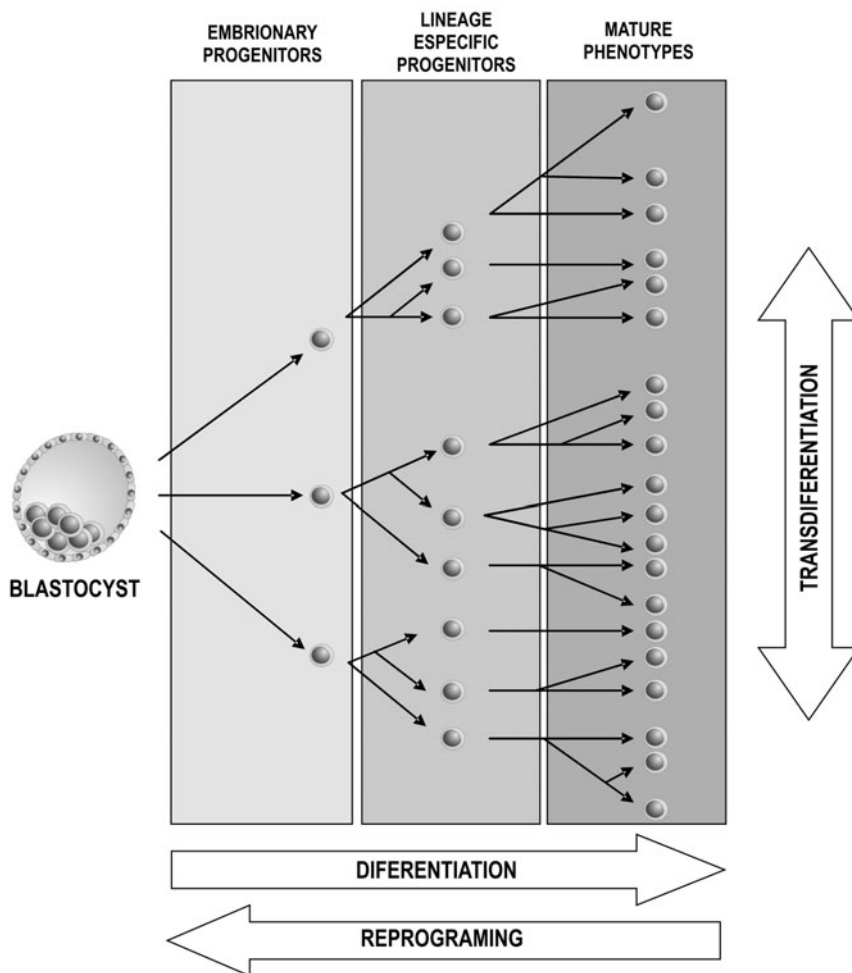
### INTRODUCTION

A clone is defined as a set of genetically identical (pluricellular or single cell) organisms produced by asexual reproduction from a single organism. The term clone derives from the greek word κλών, twig, to refer to the asexual reproduction process by which a plant can regenerate itself from a single shoot or stem. The term cloning is broadly used for any process of biological material replication, be it a cell, a pluricellular organism or a piece of DNA. However, it is important to bear in mind that being genetically identical does not necessarily imply having an identical phenotype, since the latter is also dependent on elements which the genome does not code for. A neuron, an egg cell and a tumour cell may contain exactly the same genetic information and yet they have totally different phenotypes.

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The cell is the basic unit of any living organism. All the cells our organism is composed of come from one original cell, the zygote, from which stem all the cell types present in an adult individual. This unique quality is called totipotency. During embryonic development, the zygote repeatedly divides giving rise to progenitor cells which are capable of turning into different cell types, within certain constraints, until they finally produce the whole collection of cells present in an adult organism. This process, known as cellular differentiation, seems to be unidirectional, meaning by this that a cell that acquires a given cell identity during the differentiation process cannot revert from its differentiated state and go back to being a progenitor cell (Fig. 1). However, this is not



**Figure 1.** Schematic diagram of cell differentiation during embryo and fetal development. All cell types present in an adult organism are generated from the zygote. During this process, cells gradually lose their ability to differentiate into different types of cells until they reach a stable phenotype. The reversal of the differentiation process is called cellular reprogramming. When a mature cell changes its phenotype and transforms into a cell of a different lineage, this is the phenomenon of transdifferentiation.



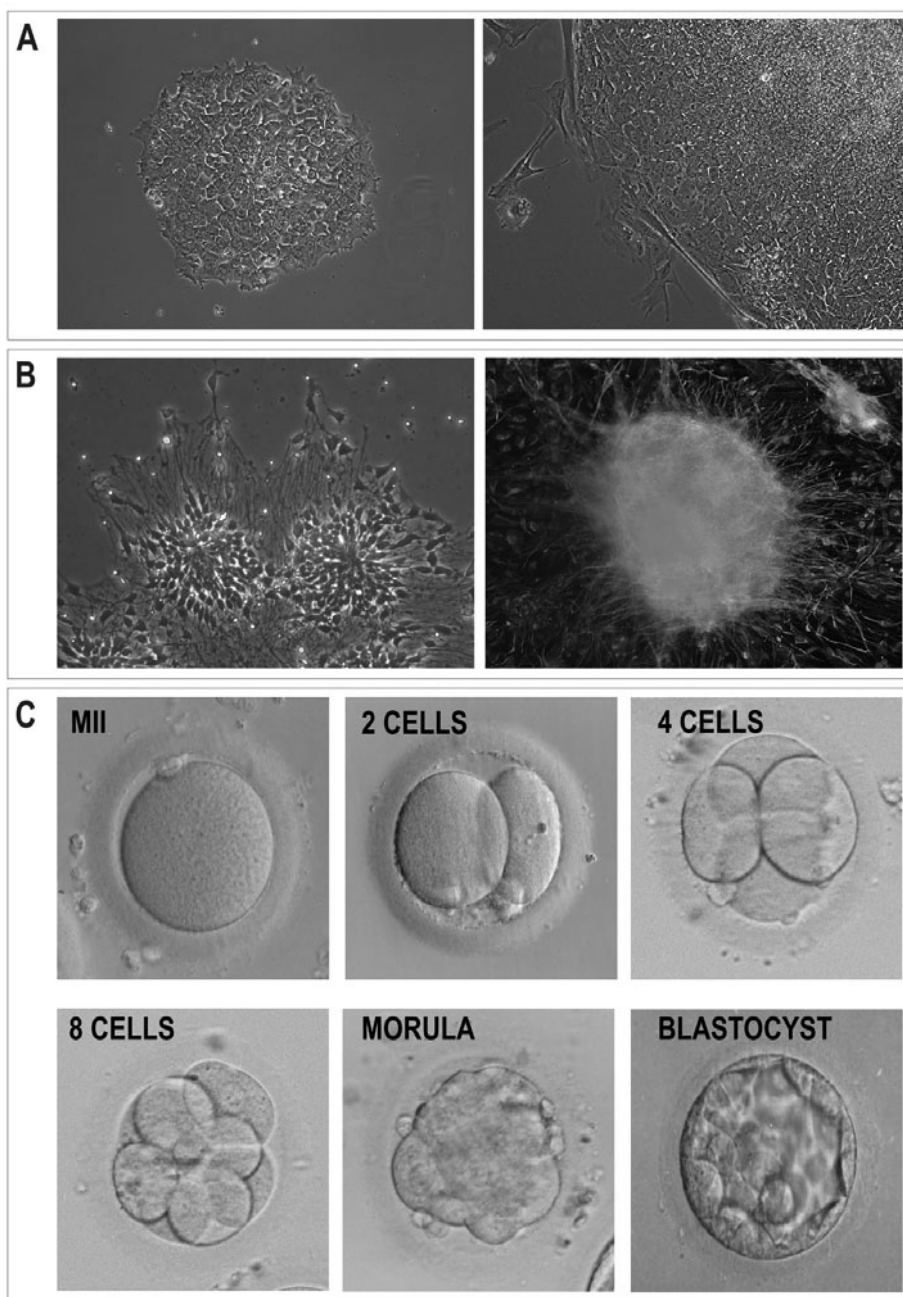
entirely true. The cells in an organism undergo stable changes during morphogenesis, but those changes are not irreversible. Pioneering work carried out by Briggs and King (1960) and Gurdon (1962) on amphibians demonstrated that the nucleus of a somatic cell, when re-introduced into an enucleated egg cell, can govern embryonic development and generate a complete adult individual.<sup>1,2</sup> A somatic nucleus introduced into an ovum becomes a zygote, a totipotent cell capable of generating all the cell types present in an adult organism. So, a process of nuclear reprogramming has taken place.

The technique of nuclear transfer proved the genetic expression programme of the somatic nucleus to be flexible and, on the other hand, that it constitutes a powerful biological tool with several applications. In 1981 Evans and Kaufman succeeded in deriving cell lines from a mouse blastocyst. These cells were named “EK cells” and they were what we current know as embryonic stem cells.<sup>3</sup> These embryonic stem cells are capable of differentiating into any cell-type present in an adult organism (Fig. 2A,B). This quality opens the door to regenerative medicine given that, in theory, it would be possible to use such cells to substitute dead cells or damaged tissues. Nuclear transfer techniques allow the generation of isogenic embryonic stem cells, genetically identical to the patient, thus avoiding the risk of immunological rejection. This strategy is commonly known as therapeutic cloning.

## USE OF AUTOLOGOUS CELLS IN REGENERATIVE MEDICINE

In 1998 stem cells were derived from a human embryo for the first time, creating a surge of expectation, due to the possible use of these cells in human cell therapy.<sup>4</sup> Their use in regenerative medicine poses, nonetheless, a huge challenge, since there is a great gap between speculations on their therapeutic usefulness and their practical use. Animal models have provided valuable information concerning possible applications of stem cells as well as about their limitations. Cells derived in vitro from embryonic stem cells have shown their effectiveness in several disease models, such as those for spinal cord lesions, Parkinson’s disease or myocardial infarction. Even though embryonic stem cells can constitute a renewable source of tissue for regenerative therapies, the question of histocompatibility is an important biological barrier to their therapeutic use. To mitigate this, it has been proposed that cell banks be created containing all the lines required to cover all the haplotypes needed for therapeutic use. The number of lines required to achieve this objective is a controversial issue. The most optimistic estimates consider around 200 lines to be sufficient for clinical applications, based on analysis HLA diversity in local populations.<sup>5,6</sup> Those estimates deem partial HLA matching acceptable, they take into account only HLA-A, HLA-B, HLA-DR and blood-type, and therefore require immunosuppressive treatment to avoid rejection. Other studies consider immunogenicity of differentiated cells derived from embryonic stem cells to be low, suggesting that total matching might not be necessary.<sup>7</sup> However, later studies have provided evidence of normal MHC expression and of immunological rejection of cells derived from embryonic stem cells.<sup>8-10</sup> Therefore, higher HLA matching is desirable, although this would require storage of thousands of cell lines, which poses a major technical challenge. The alternative to the creation of banks would be isogenic cell transplantation, that is to say, transplantation of cells derived from the patients themselves.

Therapeutic cloning consists of the generation of embryonic stem cells by somatic cell nuclear transfer. These are isogenic cells and there is complete HLA matching, so



**Figure 2.** A) Human embryonic stem cells (left panel) and human iPS cells (right panel). Both cell lines are morphologically indistinguishable. B) Neural progenitors derived from embryonic stem cells with MAP-2 (red) and nestin (green) immunofluorescence. C) Embryos generated from a human oocyte through parthenogenesis (we thank the CER Medical Institute, Argentina, where these photographs were taken). A color version of this figure is available online at [www.landesbioscience.com/curie](http://www.landesbioscience.com/curie).

immunosuppressive treatment is not required. Recent studies carried out on a murine model of Parkinson's disease found no rejection of isogenic cells derived by nuclear transfer. Allogenic transplantations, on the contrary, showed considerable immunogenicity and low cell survival rates.<sup>11</sup> So, allogenic transplantations had no therapeutic effect while isogenic cells maintained their therapeutic effect for periods longer than nine weeks. Although the use of partially histocompatible cells in combination with an immunosuppressive treatment is still a viable option, the use of isogenic cells dramatically increases the therapeutic effectiveness of cell transplantation. However, when compared to organ transplantation, data concerning immunological rejection of cell transplantations is still scarce, since they are usually performed under potent immunosuppressant drug regimens.

The possibility of using nuclear transfer technology to create embryonic stem cells for therapeutic purposes has triggered intense ethical debate. Specifically, there is controversy over the use of human oocytes in research and over the creation and destruction of embryos with potential to develop into an individual, among other issues related to research on human embryos and/or gametes. This has led to the search for alternatives to therapeutic cloning by which to obtain autologous pluripotent cells. Adult stem cells offer an interesting alternative to the use of embryonic stem cells, as they can be isolated from different tissues of an adult individual and are therapeutically useful in some diseases. It is important to underline that adult stem cells are not the same as embryonic stem cells. There are great differences with respect to their differentiation capacity and their potential range of therapeutic applications. Adult stem cells do not exhibit pluripotency and their capacity to differentiate is partially constrained, so their therapeutic use is correspondingly limited. Although adult stem cell plasticity *in vitro* seems to be higher than predicted and in some cases remarkable differentiation abilities have been reported, the possible therapeutic applications of these cells have not yet been experimentally validated.<sup>12-14</sup>

The creation of parthenogenetic stem cells has also been suggested as an alternative to therapeutic cloning. Parthenogenesis is the mechanism by which an oocyte can develop without needing to be fertilized and it is a common method of reproduction amongst a great variety of organisms such as insects, fish, amphibians and reptiles. This process does not occur naturally in mammals although it can be artificially induced and constitutes an interesting alternative to therapeutic cloning. Activation of a nonfertilized egg in mammals brings about the generation of a pseudoembryo, called parthenote (Fig. 2C). Despite its inability to conclude embryonic development, the parthenote can be used to derive embryonic stem cells which will be compatible with the donor woman. Further, the use of parthenogenetic stem cells has been proposed to create cell banks, since this technique allows the creation of homozygotic cells, which would reduce the number of lines required to achieve HLA matching. Parthenogenetic cells have properties equivalent to those of biparental embryonic stem cells, although it is likely that their differentiation capacity will be limited to some extent due to mistakes in their genetic imprinting. The maternal genome is epigenetically modified in the ovum, resulting in the inhibition of gene expression of certain alleles, that is, certain alleles are subject to maternal imprinting. The same is true in the paternal genome. Consequently, certain genes are only expressed from the allele inherited either from the father or the mother but not from both. Imprinting is, therefore, a reciprocal phenomenon; paternal and maternal genomes are not equivalent and are both necessary for the creation of a viable embryo.<sup>15</sup> Embryonic cells derived from the parthenote only retain maternal imprinting, which could partially compromise their differentiation capacity and, thus, their possible therapeutic uses.<sup>16</sup> In addition,

parthenogenetic stem cells would only be available for women of reproductive age, as to date these cells have only been derived following the activation of a mature oocyte. So, only somatic cell reprogramming, either by nuclear transfer or direct reprogramming, would enable the production of isogenic embryonic stem cells for the whole population.

## NUCLEAR TRANSFER AND CELLULAR REPROGRAMMING

Stem cells and progenitor cells modify their phenotype in the process of differentiation and maturation, a period during which the cell modifies its programme of gene expression. This “programming” process is normally irreversible and the cell progressively evolves until it reaches a stable phenotype. During its differentiation a stem cell loses its pluripotent and multipotent nature. If, on the contrary, a differentiated cell erased its gene expression programme it would recover its pluripotency: This would be dedifferentiation or reprogramming, in which the somatic cell acquired an embryonic phenotype. This mechanism does not seem to occur naturally in adults, although it has been suggested that a differentiated cell may momentarily become multipotent in response to tissue stress (damage or disease). In this case, the dedifferentiated cell would take on an adult stem cell phenotype and would perform tissue repair functions. However, the process of reprogramming may be brought about artificially using the technique of nuclear transfer. Nuclear transfer consists in the injection of a somatic cell nucleus into a previously enucleated oocyte. As a consequence, a zygote is created which is able to develop in vitro, producing an early embryo made up of pluripotent cells. Thus, it is possible to derive pluripotent stem cells which are genetically identical to the donor cell and therapeutically useful. This process is called therapeutic cloning. If, on the other hand, the embryo obtained were to be re-implanted into a recipient uterus, then a complete individual carrying the same genetic information as the donor could be created. This application of nuclear transfer techniques is called reproductive cloning. Currently it can be carried out in the case of domestic animals and therefore is of great economic importance. Therapeutic and reproductive cloning have different goals, but both procedures are identical from a methodological point of view, so the terms do not refer to a specific methodology but rather to different applications of nuclear transfer techniques.

Studies carried out on amphibians have shown the intrinsic ability of their ovum to reprogram a somatic nucleus and provided the methodological grounds for testing this in other species. One of the most interesting observations was that the differentiation state of the donor nucleus had dramatic consequences for the reprogramming process.<sup>17</sup> When donor cells had an embryonic origin the process of reprogramming was complete: A pluripotent zygote with the ability to develop to maturity was created. On the contrary, cells derived from foetal or somatic tissues rarely succeeded in creating pluripotent zygotes able to generate mature individuals. This is the case for all the animal species in which nuclear transfer techniques have been tested and it has serious implications for therapeutic cloning and its practical application. Cloning from somatic cells is possible although the efficiency of the technique is severely affected by the advanced state of differentiation. In mammals, cloning from blastomeres (embryonic cells at the pre-implantation stage) was first achieved in the 1980s. This methodology enables the disaggregation an 8- to 32-cell embryo and clones can then be produced by transferring blastomere nuclei to the enucleated egg cells. It has been trialled in commercial production systems with the aim of amplifying the number of individuals

with a high genetic potential. However, its relatively low efficiency meant that this methodology was not further developed. Progress in mammal cloning came with the birth of Dolly, a sheep cloned by fusing a mammary gland cell with an enucleated oocyte. This demonstrated that cloning from somatic cells was possible in mammals and that the state of differentiation may be reversed to the most primitive development stage. Since Dolly's birth many animals from different species have been successfully cloned. Still, the efficiency of animal production from somatic cells is very low, with less than 5% of the embryos transferred reaching full term.

In contrast to reproductive cloning, efficiency in embryo stem cell generation from cloned embryos is similar to that of embryos produced *in vitro*. Furthermore, studies on mouse stem cells suggest that the properties and characteristics of embryonic stem cells from cloned embryos cannot be distinguished from those of cells from fertilized embryos. However in humans, stem cells have not yet been derived from cloned embryos and very few articles report on the use of nuclear transfer. This is probably due to a shortage of good quality oocytes for research. Available reports indicate that the quality of the oocyte is a determining factor for the success of nuclear transfer. So far, cloned blastocysts, a fundamental step to obtain stem cells, are only known to have been obtained twice. In both cases, only the use of oocyte from fertile women, manipulated shortly after their collection, successfully produced blastocysts. In the study reported by Stojkovic and colleagues (2005) embryonic stem cells were used as donor cells, so probably a lower degree of reprogramming was required.<sup>18</sup> On the other hand, French et al (2008) managed to produce five human blastocysts following the transfer of adult somatic cells to enucleated oocytes.<sup>19</sup> Perhaps as significant as the production of cloned human embryos is the recent success in obtaining monkey embryonic stem cells by nuclear transfer of somatic cells. These studies provide evidence that human therapeutic cloning is feasible. Nevertheless, limited availability of good quality human oocytes may remain the principal obstacle to a technical implementation of this methodology.

To date, the molecular mechanism underlying nuclear reprogramming in eggs is poorly understood. It is uncertain whether the process of reprogramming is a passive one, which occurs as a consequence of the dilution of the factors required for the maintenance of cell identity, or is active, leading to the re-establishment of pluripotency. In fact, it is likely that a combination of active and passive processes is responsible for the conversion of somatic cells into pluripotent cells. A fact, clearly demonstrated by cloning, is that neither cellular differentiation nor dedifferentiation, through cloning, are processes which alter the genomic sequence but rather they modify epigenetic marks that control the expression of the genome. These epigenetic marks are represented by DNA methylation and post-transcriptional modifications of histones. During embryonic development, DNA methylation is reprogrammed by a process of erasure and re-establishment. There are two methods by which DNA methylation occurs: Active demethylation, in which paternal DNA is demethylated rapidly without cellular division, and passive demethylation, in which a failure to maintain methylation after DNA replication leads to a decrease in maternal DNA methylation in successive cell cycles. Later, DNA methylation is re-established. Curiously, low DNA methylation level reached by the embryo during its early development is only tolerated by pluripotent cells, such as those of the germ line and embryonic stem cells. When an equivalent methylation level is induced in somatic cells, they do not survive. The enzyme(s) responsible for active demethylation during the first hours of embryo development has (have) still not been identified by researchers, while enzymes responsible for maintaining and establishing



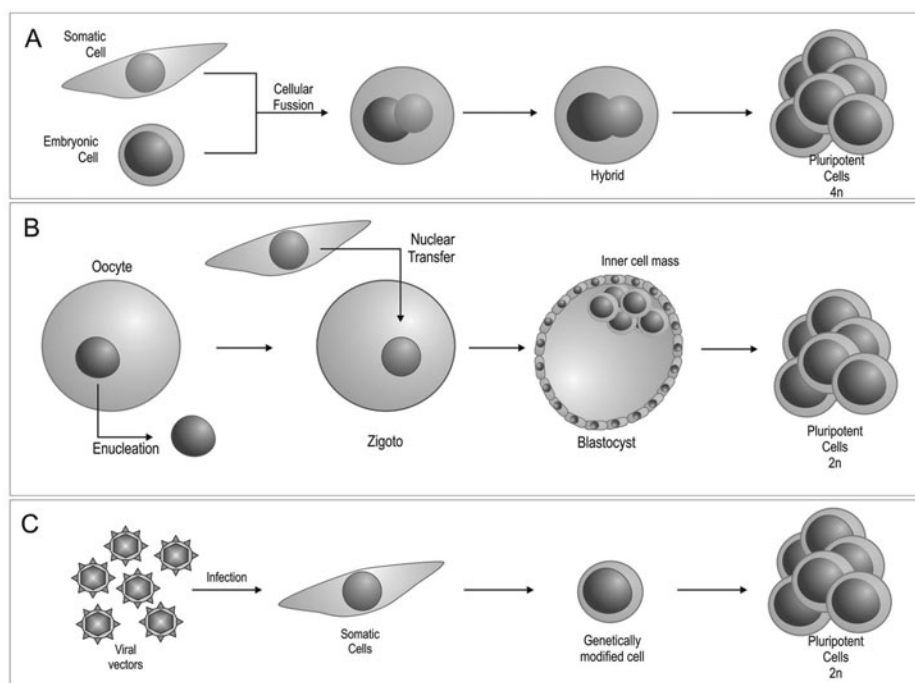
these marks are well characterized. During the cloning process genomic DNA is demethylated, although the difference between maternal and paternal genome demethylation is not reproduced. In addition, a lower level of demethylation level is achieved than that observed in fertilized embryos. These differences between clones and fertilized embryos have been suggested as the cause for incomplete reprogramming of the somatic nucleus and for the low efficiency of the technique.<sup>20</sup>

The implantation rate and the number of births are both considerably higher in embryos generated by in vitro fertilization (IVF) than in embryos reconstructed by nuclear transfer (NT). However, no notable differences have been observed concerning the development potential during the pre-implantation period to the blastocyst stage or concerning embryonic stem cell derivation efficiency. For use in therapeutic cloning, embryonic cells derived from a clone must have the same differentiation potential as cells derived from fertilized embryos. The ability to generate teratomas and to contribute to the formation of chimeras seems to show that pluripotency of these cells is not endangered. Nonetheless, abnormalities observed in cloned embryos suggest that cloned cells and biparental cells are not exactly the same and that the process of reprogramming is an incomplete one. Transcriptome analysis of murine cloned and fertilized blastocysts has found that less than 1% of the genes show differential expression, although more dramatic differences have been observed at the epigenetic level. Some epigenetic marks, such as DNA and histone methylation, seem to be substantially different in the trophoblastic cells of blastocysts.<sup>21</sup> Most of abnormalities observed in animals are related to problems with placental function. It is therefore plausible that defects observed in clones are largely due to defects in trophoblastic cells and not in the inner cell mass of the blastocysts from which embryonic stem cells are derived. Moreover, embryonic stem cells derived from cloned embryos are able to efficiently generate a viable embryo when provided with functional trophoblastic cells, using the method of tetraploid complementation of the placenta.<sup>22</sup> All in all, there is solid evidence of the reprogramming process being normal in cloned embryonic stem cells, and accordingly that these cells are equivalent to stem cells derived from fertilized embryos.

### **DIRECT CELLULAR REPROGRAMMING (REPROGRAMMING WITH NO EGG)**

Using nuclear transfer techniques it is possible to efficiently reprogram a somatic cell and to generate pluripotent cells of great therapeutic value. On the other hand, therapeutic cloning has raised considerable controversy due to the fact that it requires the generation and destruction of a human embryo. Nevertheless, it is possible to reverse the differentiation process without using an egg cell and thereby avoiding the need to produce embryos. For this, an adult cell has to erase its gene expression programme and acquire a more primitive and pluripotent state without turning into a zygote. So far, only reprogramming through cell fusion and transgenesis have proven to be methods capable of dedifferentiating somatic cells without any need for an egg cell (Fig. 3).

In 1965, cell fusion techniques were developed by Henry Harris and have later been used for a great number of applications, including production of monoclonal antibodies and X chromosome inactivation studies. Moreover, the generation of somatic hybrids provided important information concerning the acquisition of cell identity. It was observed that when two different cell types are fused, the expression of constitutive genes is not



**Figure 3.** Strategies for somatic cellular reprogramming ( $2n$  = diploid,  $4n$  = tetraploid). A) Reprogramming through cell fusion. B) Reprogramming through nuclear transfer (ICM = inner cell mass). C) Pluripotency induction.

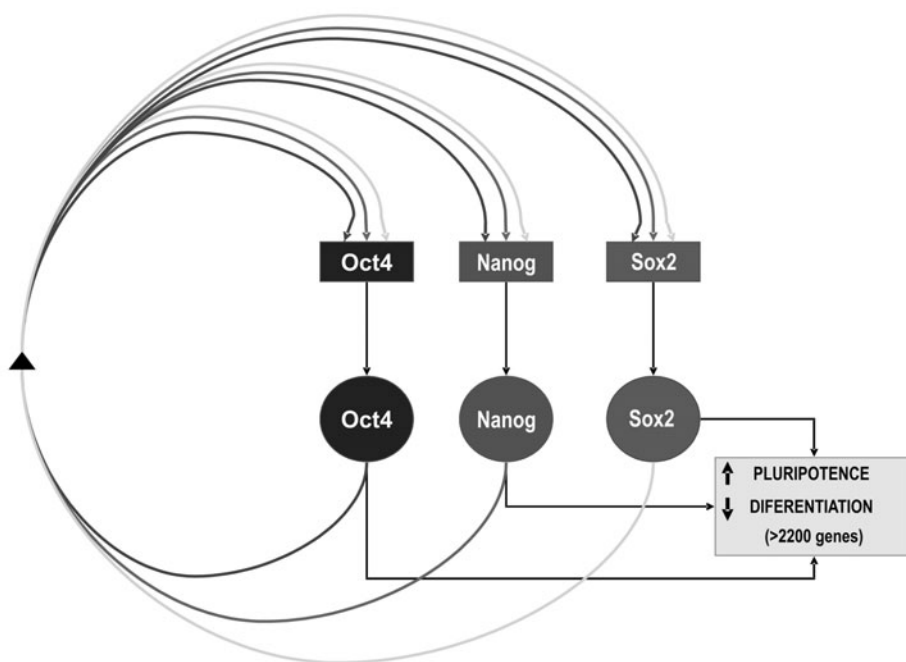
affected. Yet, generally the expression of genes expressed by only one of the two cell types forming the hybrid is altered. This mechanism is widely known as extinction. On the other hand, when a somatic cell is fused with a cell derived from an embryonal carcinoma, the resulting hybrid was found to acquire a phenotype similar to that of an embryonic cell and the inactive X chromosome activated.<sup>23</sup> This deep phenotypical change suggests dramatic changes at epigenetic level and in gene expression. Furthermore, when an embryonic stem cell is fused with a somatic cell, a thymocyte, the resulting cell acquires characteristics of an embryonic stem cell and keeps its pluripotency.<sup>24</sup> It has been shown that the somatic nucleus re-activates genes which are associated with the pluripotent state while the expression of somatic specific genes is extinguished. Accordingly, not only a phenotypical change but a mechanism of cellular reprogramming has taken place. Another example of this mechanism was seen in the experiments carried out by Tada et al (1997) in which somatic and embryonic germinal cells were fused.<sup>25</sup> Resulting hybrids again acquired an embryonic phenotype but, in addition, parental imprinting in the somatic nucleus was lost, a phenomenon which does not occur in hybrids created from embryonic stem cells.

Hence, fusion techniques make it possible to change cell identity and also provide new evidence of the plasticity of the somatic nucleus. However, the most important discovery is that the somatic nucleus does not trigger differentiation of the pluripotent nucleus; on the contrary, the somatic nucleus itself is reprogrammed. Reprogramming through cell fusion reveals that the maintenance of pluripotency in a primitive cell hides



an active mechanism capable of erasing the gene expression programme of a somatic cell. The process by which a somatic cell acquires a stable phenotype, a cell identity, seems to be different from the mechanism of acquisition and maintenance of pluripotency.

The maintenance of pluripotency by an embryonic cell depends upon a set of extrinsic and intrinsic influences. Specifically, it depends on growth factors, cell-cell and cell-matrix interactions, transcription factors, epigenetic control over gene expression, etc. In addition, some of the intrinsic molecular elements involved in pluripotency have been identified and three transcription factors seem to have a central role: Oct-4, Nanog and Sox-2. These three genes regulate their expression reciprocally and control the expression of many genes relevant to embryonic development and cell differentiation (Fig. 4). Furthermore, they are indispensable for maintaining pluripotency, they are specifically expressed in the inner cell mass of embryos and in embryonic stem cells, and their expression rapidly decreases during the differentiation process. It is possible to speculate on these three genes being part of the active reprogramming mechanism observed during cell fusion experiments. It has been shown that the somatic nucleus starts to express Oct-4 after fusion with an embryonic stem cell. The mechanism by which Oct-4 is re-activated is unknown, although it is possibly mediated by the Oct-4, Nanog and Sox-2 expressed by the embryonic nucleus, given that these factors are capable of inducing Oct-4 expression by interacting with its promoter.<sup>26</sup> However, other molecular elements such as transcription factors, chromatin remodelling factors and microRNAs may also play a role in the re-activation of genes associated with pluripotency. Reducing the mechanism of



**Figure 4.** Central pluripotency circuit. Oct4, Nanog and Sox2 reciprocally regulate their gene expression and control the expression of over 2200 genes involved in the processes of differentiation and pluripotency.

maintenance of pluripotency to a basic circuit of just three genes is likely to be a simplification of a more complex process. Indeed, a new factor called Ronin has recently been described which seems to be necessary for murine stem cell pluripotency and its function is probably associated with epigenetic control over gene expression.<sup>27</sup>

Given that Oct4, Nanog and Sox2 are capable of regulating their own gene expression, it is reasonable to suppose that the molecular mechanism of pluripotency is capable of self-regulation and of maintaining its status as long as “extrinsic” requirements are met. So, if there really is a self-regulating mechanism capable of actively inducing pluripotency, then it is possible to imagine that transferring that mechanism to a somatic cell would make the reprogramming process feasible, as long as cells are provided with the right culture conditions. It is therefore crucial to identify the molecular elements which constitute that mechanism. Since the reprogramming mechanism is considered to be a very complex process involving a great number of elements, “egg-independent” cellular reprogramming has been a long-term objective for the scientific community. That is why the experiments carried out by Takahasi and Yamanaka (2006) which led to the generation of induced pluripotent stem (iPS) cells have had such a great impact: Besides constituting a new therapeutic tool, those experiments create a new paradigm for the process of cellular reprogramming.<sup>28</sup> These researchers started by selecting a group of genes that were only expressed by stem cells. Then, incorporating these factors into mouse fibroblasts, using viral vectors (Moloney virus) with high transduction efficiency, they observed the creation of cells which expressed genes that are characteristic of embryonic stem cells. Finally, they started to sequentially subtract the various factors until they reduced their list down to four indispensable factors, which were sufficient to induce reprogramming: Oct4, Sox2, Klf4 and c-myc. Since their seminal work, Yamanaka et al as well as other research groups, have reported improvements in the methodology which have made it possible to produce iPS cells which are very similar to embryonic stem cells: They are pluripotent, have the capacity to generate teratomas and contribute to the formation of chimeras at germ line level.<sup>29</sup> Moreover, the use of this technology for therapeutic treatment has been demonstrated on mice in models of Sickle Cell anaemia and Parkinson’s disease.

Given that Oct4 and Sox2 seem to be basic elements for the maintenance of pluripotency, their involvement in the process of reprogramming comes as no surprise. However, the role of oncogenes c-myc and Klf4 is still unclear. In particular, c-myc does not seem to be essential in the reprogramming process although it enormously increases its efficiency. However, chimeric mice generated from iPS cells very frequently develop tumours, and this is fundamentally due to the re-activation of c-myc. On the other hand, it has been possible to reprogram somatic cells without using oncogenes. Indeed, the first human iPS cells were derived in the absence of oncogenes, using a different set of factors: Oct4, Sox2, Nanog and Lin28. These cells have the capacity to differentiate *in vitro* and to generate teratomas and they have a transcriptome that seems to be equivalent to that of human embryonic stem cells.<sup>30</sup> Furthermore, human cell reprogramming has been achieved using the same combination of genes originally used for mouse cells, which seems to suggest that the reprogramming process is very similar in both species.

The mechanism by which these factors are capable of reprogramming a somatic cell is unknown. Oct4 and Sox2 are believed to be responsible for the re-activation of the central pluripotency circuit (Oct4, Nanog and Sox2), which in turn is thought to induce reprogramming, in a similar way to the mechanism that takes place during reprogramming by cell fusion. On the other hand, the process of reprogramming by transgenesis takes a long time, 3 to 4 weeks, and requires repeated cycles of cell division to achieve complete

reprogramming. It is possible that during successive mitotic divisions, cells lose their ability to maintain their epigenetic status, and that this facilitates their transition to a pluripotent phenotype. This suggests that the process of reprogramming is partly stochastic, as it does not take place in the reverse way to the process of differentiation and therefore, reprogramming and dedifferentiation are not equivalent mechanisms. Furthermore, clonal expansion of infected cells provides evidence of the stochastic nature of the process, given that only a small proportion of the cells manage to reprogram themselves and become pluripotent.<sup>31</sup>

Regenerative medicine gains one more therapeutic tool with iPS cells. However, their potential applications are limited by the fact that the viral vectors used need to stably integrate into the host genome. These integrations occur randomly and may cause insertional mutagenesis, which can eventually lead to cancerous transformation. In any case, iPS cells prove that adult somatic cells can be reprogrammed into pluripotent cells using just three or four factors. The challenge is now to achieve the same transformation using factors or substances that do not permanently modify the genome. Among the strategies being studied is the use of genetic vectors which do not integrate into the genome, such as plasmids,<sup>32</sup> cre-excisable viruses,<sup>33</sup> and piggy bac transposition system.<sup>34</sup> Nevertheless, genetic approaches may lead to unpredicted genetic modifications. In a recent report, recombinant proteins were used to generate mouse IPS cells. The reprogramming factors Oct4, Sox2, Klf4 and c-Myc were fused to C-terminal poly-arginine tail in order to facilitate penetration through the plasma membrane.<sup>35</sup> Another possibility which seems to have wider support is reprogramming using chemical substances. In relation to this, it has been demonstrated that chemicals which induce DNA demethylation or an overall increase in histone acetylation have a positive effect in virus mediated mouse cellular reprogramming, increasing the reprogramming efficiency by a factor of 100. Further, with neural progenitors obtained from mouse fetuses which express endogenous Sox2, Shi et al have managed to replace Oct4 in the virus cocktail with BIX-01294, an inhibitor of histone methylation.<sup>36</sup> These molecules target the epigenetic machinery of the cell, and probably they facilitate the epigenetic changes required for reprogramming. Nevertheless, a recent report by Ichida et al describes a new molecule, RepSox, that do not inhibit any epigenetic enzyme and can greatly enhance the efficiency of the reprogramming process. RepSox is an inhibitor of the transforming growth factor- $\beta$  (TGF- $\beta$ ) signal. This inhibition leads to Nanog overexpression and can replace Sox2 in the reprogramming cocktail.<sup>37</sup> Moreover, IPS cells generation has been reported with a single gene, OCT4, and small molecules.<sup>38</sup> These recent findings suggest that exclusively chemically mediated reprogramming may be achieved in the not too distant future.

## CONCLUSION

It is remarkable that, 50 years on, the molecular mechanism that rules the reprogramming process still eludes scientists. Nevertheless, some of its molecular elements have been identified and new tools for genome and epigenome analysis have made it possible to place them in the correct molecular context. On the other hand, the development of iPS cells has led to an important qualitative change in the field of cellular reprogramming and regenerative therapy, given that it overcomes some of the ethical barriers associated with therapeutic cloning. All this has given the study of cellular reprogramming a great boost and has turned into a field of extraordinary social and economic relevance.

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## CHAPTER 19

# ADVANCES IN STEM CELL THERAPY

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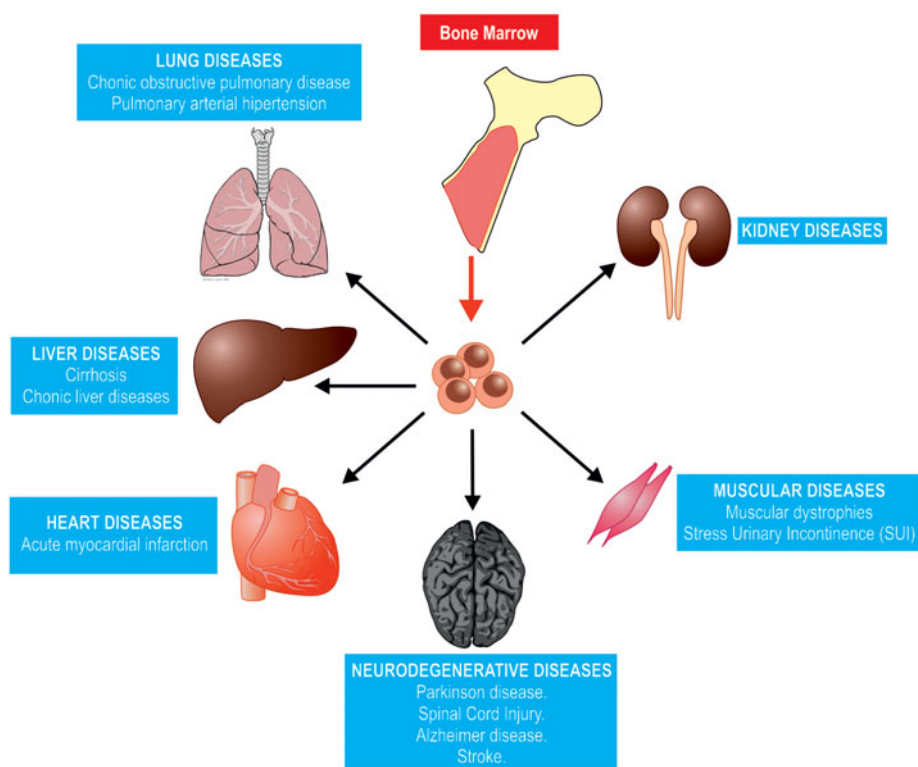
**Abstract:** Since the beginning of stem cell biology, considerable effort has been focused in the translation of scientific insights into new therapies. Cell-based assays represent a new strategy for organ and tissue repair in several pathologies. Moreover, alternative treatment strategies are urgently needed due to donor organ shortage that costs many lives every year and results in lifelong immunosuppression. At the moment, only the use of hematopoietic stem cells is considered as the standard for the treatment of malignant and genetic bone marrow disorders, being all other stem cell applications highly experimental. The present chapter tries to summarize some ongoing approaches of stem cell regenerative medicine and also introduces recent findings from published studies and trials conducted in various tissues such as skeletal muscle, liver and lung.

## INTRODUCTION

Stem cells possess the capacity to proliferate extensively as in case of injury or during development. Embryonic stem cells (ESCs) are the most potent stem cells, capable of unlimited growth in culture and able to differentiate to all types of human tissue, being considered pluripotent. On the other hand, adult stem cells are multipotent because they have a limited capacity of self-renewal and they can only generate differentiated lineages appropriate for the tissue from which they are derived. Adult stem cells comprise, at least, 3 different groups: Bone marrow-derived stem cells, the circulating pool of progenitor cells, which, at least in part, are derived from the bone marrow (BM), and tissue resident stem cells; these tissue stem cells, which have been demonstrated in different organs, are considered to be unspecialized cells showing the capacity for unlimited self-renewal and the ability to produce more differentiated and committed cells (Fig. 1).

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**Figure 1.** Differentiation of bone marrow adult stem cells to different tissues and possible application in the therapy of diverse pathologies.

Thus, one of the main sources of stem cells is the bone marrow, giving haematopoietic precursors and three different groups of cells (Mesenchymal stem cells-MSCs, Endothelial progenitor cells-EPCs and other population that includes monocyte precursors, T and B-cell precursors, CD14 cells, ...). Further emergent stem cells are iPSCs (induced pluripotent stem cells) that are dermal fibroblasts genetically engineered to behave like human embryonic stem cells (hESCs). Finally, a relative new source for progenitor cells is umbilical cord blood, its cells are easily obtained and despite not being available in as large quantity as other cells have the potential to develop into multiple lineages without posing the ethical questions inherent to embryonic cells.

However, before the expansion of the clinical use of stem cells it has to be taking into account several issues: First of all, the potential risk of a stem cell intervention cannot be justified if an efficient pharmacological therapy already exists; secondly, it is necessary to define the most adequate type of cell or cells for each application. It is also important to keep in mind that while prior basic research in animals is needed to test these therapies, these models cannot mimic all the aspects of human pathology. Finally, it is necessary to determine the biological mechanisms underlying how any observed positive effects are different than those found with other therapies such as immunomodulation.<sup>1</sup>

Currently, the majority of studies evaluating the clinical use of progenitor cells are Phase I and Phase II trials with the purpose of determining the safety and the efficacy



**Table 1.** ISSCR guidelines for the clinical translation of stem cells

Responsibility for conduct	Recommendations 1-2	Creation of review committees formed with scientific and ethical expertise in order to ensure the establishment of care standards according to ISSCR guidelines.
Cell processing and manufacture	Recommendations 3-10	In the case of allogeneic use, donors should give written informed consent covering issues like cell storage, privacy or disease screening. Components of animal origin should be avoided. Development of common reference standards to ensure minimal acceptable changes during cell processing as well as optimum product quality (Adherence to GMP procedures).
Preclinical studies	Recommendations 11-19	Sufficient preclinical studies in relevant animal models are needed to make stem cell-based clinical research so as to ensure safety, efficacy and low toxicity. Cells should be rigorously characterized to assess the potential toxicity as well as the risk of tumorigenicity and the interaction with drugs and immunosuppressants.
Clinical research	Recommendations 20-33	Researchers should address to the patients the risk of stem cell-based interventions and provide with clarity all the informations concerning the applied therapy. Participants should give informed consent. Patients should be monitorized and adverse events must be reported. Stem cell-based approach must be clinically competitive to existing therapies. Positive and negative results should be published.
Stem cell-based medical innovations	Recommendation 34	All the studies should have a written plan for the procedure including adverse events.
Considerations of Social Justice	Recommendations 35-39	Collaborations between researchers and institutions are recommended as well as fair access and ethics.

of a particular procedure. In view of the increasing number of studies with stem cells, the ISSCR (International Society for Stem Cell Research) published a professional guide in 2008 for researchers who wished to translate basic stem cell research into clinical applications.<sup>2</sup> This document contains 40 recommendations, and is summarized in Table 1.

## STEM CELLS AND NEURODEGENERATIVE DISEASES

Neurodegenerative diseases include a wide range of acute and chronic alterations in which neurons and glia within the brain and spinal cord are lost. In acute cases, such as ischemic stroke or spinal cord injury, different types of neurons and glial cells die within

a restricted brain area over a short period of time. In chronic cases, there is a selective loss of a specific cell population such as dopamine neurons in Parkinson's or a degeneration of varied types of neurons over a period of several years, such as in Alzheimer's. Although some clinical trials using stem cells to treat neurodegenerative disorders have already been performed, no stem cell therapy has yet been proven beneficial for any condition. However, effective cell replacement might be achieved by inducing endogenous stem cells in the adult CNS to form new neurons and glial cells. Besides these mechanisms, grafted stem cells and their derivatives could also induce functional improvement by releasing neuroprotective therapeutic molecules or by modulating inflammation.

### **Stem Cell-Based Therapies for Parkinson Disease**

The transplantation of dopamine rich human foetal mesencephalic tissue from aborted human fetuses showed evidence of cell replacement in the altered areas.<sup>3</sup> Furthermore, some patients showed an alleviation of motor symptoms and their graft-derived neurons were found to be still functional after ten years, proving that there are long-term clinical benefits.<sup>4</sup> However, others exhibited modest, if any, clinical improvement.<sup>5</sup> In some cases the patients suffered from post-operative graft-induced dyskinesias (GIDs) which have been reported in, at least, two different trials.<sup>6,7</sup> Regardless of the efficacy of treatment using ESCs, ethical issues surrounding their use make it necessary to find other sources of neurons. For example, dopaminergic neuroblasts for use in preclinical transplantation have been generated in vitro from not just ESCs<sup>8</sup> but also BM-derived stem cells<sup>9</sup> taken from various species, including humans. Unfortunately, some fundamental problems standing in the way of successful clinical translation, have not yet been solved for human stem cell-derived dopaminergic neurons; for example, the use of ES cell-derived dopaminergic neuroblasts present the risk of tumour formation, a problem that could be solved using neuroblasts made from iPSCs. This would eliminate many ethical concerns associated with ESCs, and would also help to avoid immune responses. Moreover, cell replacement should be combined with neuroprotective therapy such as the use of modified cells that secrete trophic factors like GDNF (glial cell-derived neurotrophic factor). In order for cell transplantation procedures to be seen as viable alternatives they should offer benefits that other treatments can not give us, such as the amelioration of motor symptoms without significant side effects.

### **Stem Cell-Based Therapies for Spinal Cord Injury**

Different types of stem cells have been implanted in animal models of spinal cord injury resulting in improvement of functional outcomes.<sup>10-12</sup> Also, stem cell-based approaches using umbilical cord blood, bone marrow-derived haematopoietic stem cells (HSCs) and MSCs have already been used to treat patients with spinal cord injury, with claims of partial recovery.<sup>10</sup> Based on these findings, different attempts for treating spinal cord injury in humans have been made. Encouraging results, such as improved walking ability and recovery of sensory perception, have been reported.<sup>13</sup> Currently, a clinical trial is underway in Ecuador, supported by Prime Cell Therapeutics to test the efficacy of transplanting autologous BM-derived stem cells into the injured spinal cords of 25 patients. More recently, the results of a Phase I/II study in which 297 patients were treated with mononuclear cells (MNCs) injected via lumbar puncture concluded that the outcome of therapy was influenced by both the amount of time between injury and treatment, and

CD34+ cell number. They also found that one-third of the participants showed perceptible improvements in their neurological status. Furthermore, examination of adverse effects revealed the procedure to be safe.<sup>14</sup>

Moreover, the first Phase I clinical trial testing the use of ESC-derived oligodendrocyte precursor cells (OPCs) is planned by the US Company, Geron. Patients with thoracic spinal cord injury will be immunosuppressed for 2 months following OPCs transplantation and then tested for recovery of sensory perception and lower extremity motor function. This initial trial of human ESCs-derived stem cells raises concerns about the risk for tumour formation, which is difficult to assess in a preclinical xenograft situation. There are several limitations with this approach, including implanted cells that were often poorly characterized, preclinical evidence of efficacy being insufficient in some cases, and the reported benefits appearing in patients also receiving physiotherapy.<sup>1</sup>

### **Stem Cell-Based Therapies for Amyotrophic Lateral Sclerosis**

At this moment there is no effective pharmacological treatment for ALS. However, motor neurons have been generated in vitro from stem cells of various sources such as ESCs<sup>15</sup> and human forebrain.<sup>16</sup> Recently, the US Company Neural Stem has received FDA approval for a clinical trial in which 12 patients with ALS will be treated by injection of human fetal-derived neural stem cells (NSCs) into the lumbar region of the spinal cord, where it is hoped they will exert a neuroprotective effect. Fortunately, HSCs transplantation as well as the delivery of MSCs to alter the inflammatory environment has already reached the clinic. Although allogenic HSCs have been transplanted intravenously in 6 ALS patients<sup>17</sup> there was no evidence of any clinical benefit. However, donor-derived cells were found to localize to the sites of pathology, which suggest that HSCs are particularly suitable for delivering therapeutic molecules. In another study, 9 patients received intraspinal injections of autologous MSCs<sup>18</sup> these findings indicated that the lumbar injection of human MSCs dampened inflammation and reduced both motoneuron loss and functional impairment. The same research group has also recently published the results of a Phase I clinical trial involving 10 patients who received MSCs that were isolated from bone marrow, suspended in autologous cerebrospinal fluid (CSF) before being surgically transplanted into the spinal cord at the thoracic level.<sup>19</sup> This trial found no immediate or delayed transplant-related toxicity. Furthermore, all clinical, laboratory, and radiographic evaluations of the patients showed no serious transplant-related adverse events, confirming that transplantation of MSCs into the spinal cord of ALS patients is safe. Haematopoietic stem cells have also been used in cervical intraspinal injections showing amelioration of symptoms in 9 of 13 patients.<sup>20</sup> Currently, a Spanish group from Hospital Universitario Virgen de la Arrixaca is performing a Phase I/II study using BM-MNCs as well as laminectomy (ClinicalTrials.gov Identifier: NCT00855400). The above studies reported clinical benefits. However, since the preclinical data (safety, dosage, long-term survival, postmortem biopsy) are insufficient and clinical evidence of improvement is weak, more preclinical studies are needed prior to the development of further clinical applications.

### **Stem-Cell Based Therapies for Alzheimer Disease**

Stem cell-based cell replacement strategies for Alzheimer disease (AD) are currently very far from clinical application. Up to now, studies have indicated there is deficient maturation of new neurons in AD brains.<sup>21</sup> However, preclinical studies have demonstrated

that basal forebrain grafts of fibroblasts produce nerve growth factor (NGF), counteracting cholinergic neuron death, stimulating cell function and improving memory in animal models of AD.<sup>22</sup> Furthermore, basal forebrain grafts of NGF-secreting fibroblasts have also proven to be of some benefit to patients with AD in an open-label trial<sup>23</sup> and basal forebrain implantation of encapsulated retinal pigment epithelial cells releasing NGF is currently being tested in 6 patients with AD by the Danish company NsGene.

### **Stem Cell-Based Therapies for Stroke**

Some initial clinical trials on the delivery of stem cells in stroke have been completed. The first clinical trial was a Phase I study in which neuroteratocarcinoma cells (NT2N) were transplanted in 12 stroke patients.<sup>24</sup> The autopsy of one patient who died due to other causes revealed a population of grafted immunoreactive cells with no signs of inflammation or neoplasia. Moreover, 6 patients showed improvement according to European Stroke Scale (ESS) compared to baseline measurements taken before transplantation. However, while there were no statistically significant trends compared to the 4 controls, five years after the surgery no adverse events were reported. After these results, the same authors presented in 2005 a Phase II randomized trial<sup>25</sup> testing the safety, feasibility and effectiveness of NT2N cells. In other study, no cell-related adverse events were observed and no substantial clinical improvements were detected in patients with an ischemic lesion in the territory supplied by the middle cerebral artery (MCA) after intravenous injection of autologous MSCs.<sup>26</sup> The significance of these results is unclear, particularly when taking into account the small sample size, the reports of some adverse effects such as epilepsy and the fact that long-term follow-up will be required to determine the tumorigenic potential of the cells. The company ReNeuron (UK) is planning a clinical trial in stroke patients involving transplantation of clonal, conditionally immortalized neural stem cells (NSCs) isolated from human fetal cortex (ReN001 cell line). Moreover, two clinical studies are currently recruiting patients for autologous cell transplantation. The aim of the first study is to determine the effects of an infusion of autologous CD34+ subset of BM-derived stem cells into the MCA of patients who have suffered acute total anterior circulation syndrome (ClinicalTrials.gov Identifier: NCT00535197). The second trial will be conducted in Brazil and will evaluate the consequences of intra-arterial injection of autologous BM-MNCs during the acute and sub-acute phases of ischemic cerebral infarct within areas served by the MCA territory (ClinicalTrials.gov Identifier: NCT00473057).

Besides the above studies other registered trials are ready to start, with most of them currently recruiting participants. One such study is treating chronic stroke patients using CD34+ cells administered via intercerebral injection (ClinicalTrials.gov Identifier: NCT00950521), a second study in ischemic stroke patients less than 6 weeks following stroke using autologous MSCs administered via intravenous injection (ClinicalTrials.gov Identifier: NCT00875654) and, finally, a study treating patients 24-72 hours following acute ischemic stroke using autologous BM-MNCs administered with a Peripheral IV device (ClinicalTrials.gov Identifier: NCT00859014). However, it is important to remember that the above studies are only Phase I/II trials focused narrowly on testing the safety and feasibility of different cell types and methods of infusion. Many issues still remain before stem cell-based therapy can advance to clinical trials for the treatment of stroke. At time, stem cell-based treatments that act by neuroprotection, modulation of inflammation and enhancement of angiogenesis seem to be closer to application in patients.

## STEM CELLS AND MUSCULAR DISEASES

It has been known for more than a century that skeletal muscle, the most abundant tissue in the body, has the ability to regenerate new muscle fibers after being damaged by injury or diseases. In 1961 Alexander Mauro<sup>27</sup> observed mononuclear cells between the basal lamina surrounding each muscle fiber and the plasma membrane surrounding the muscle fiber itself and named them satellite cells (StCs). These cells are still considered the primary stem cells responsible for skeletal muscle regeneration. These cells also contribute to the postnatal muscle fiber growth and until recently only StCs, (and, to a very minor extent, CD133<sup>+</sup> cells) have been used in human clinical trials to treat muscular pathologies, including several forms of muscular dystrophy, heart failure associated with myocardial infarction (HFMI), and stress urinary incontinence (SUI).

### Muscular Dystrophies

In 1990 the first StCs transplant was reported in a 9-years-old boy affected by Duchenne muscular dystrophy (DMD), which demonstrated that the use of these cells was safe and they could contribute to endogenous dystrophin production.<sup>28</sup> After this first approach eleven clinical trials in DMD patients were conducted using intramuscular injections of StCs.<sup>29</sup> Although the study found no adverse effects, 15% of patients showed some improvement muscle strength and new dystrophin production was found in many but not all the cases. However, none of the treated patients showed any significant clinical benefits.<sup>30</sup> This was determined to be because intramuscular injected cells are able to distribute locally but not through the circulation. Immune responses have also been detected, even if the major histocompatibility locus coincides and it has been reported the death of the most of the StCs in the first 72 hours after injection.<sup>31</sup> However, a Phase I clinical trial has provided encouraging results,<sup>32</sup> and in a report of the outcomes of the first muscle derived CD133<sup>+</sup> transplant<sup>33</sup> using autologous cells in 8 boys with DMD, no adverse events were described.

### Stress Urinary Incontinence

Stem cell-derived myoblasts have also been used as cell therapy for individuals with SUI. This treatment has been performed using two different strategies: the injection of autologous myoblasts to improve the sphincter tone or the injection of autologous myoblasts and fibroblasts (isolated from the same biopsy) to improve both sphincter tone and treat the mucosa atrophy. All of the studies published until present are nonrandomized, open studies, demonstrating clinical improvement in most of the patients treated.<sup>34,35</sup> While the onset of improvement appears six months after cell injection, the benefit lasts for a minimum of 12 months.<sup>36</sup> Unfortunately, these results have not yet been confirmed in a randomized study.

In conclusion, the main limitation of StCs is their loss of pluripotency upon culture and their inability to cross the vessel wall for systemic delivery. A Phase I clinical trial is planned for the end of 2010 using donor-derived MABs which are currently the best candidates for cell therapy based treatment for muscular dystrophy. MABs are vessel-associated progenitors that express early endothelial markers when isolated from the embryo, and pericyte markers when isolated from postnatal tissues. MABs

are also able to cross the vessel wall and are easily transduced with lentiviral vectors currently used in preclinical models of cell therapy for muscular dystrophy.

## STEM CELLS AND LUNG DISEASES

At present, allogeneic lung transplantation is considered the only treatment option for progressive and irreversible lung diseases. Unfortunately, the number of donors is still insufficient. The capability of stem cells to locally repair damaged tissue offers the possibility for cell-based treatment. In a study, cystic fibrosis patients were treated with BM-derived MSCs mixed with primary airway epithelial cells that restored the altered expression of the cystic fibrosis transmembrane conductance regulator (CFTR)<sup>37</sup> this factor is defective in patients with cystic fibrosis. These results provide proof of principle for using MSCs for CF therapy. Moreover, bronchoalveolar stem cells (BASCs) the endogenous stem cells of the respiratory tract have already been isolated, identified and located at the bronchoalveolar ductal junction.<sup>38</sup> However, despite encouraging *in vivo* results using animal models, that suggest the possibility of using stem cells in the therapy of lung diseases, the majority of the authors recommend caution before their use in humans.

### Severe Chronic Obstructive Pulmonary Disease

Concerning Severe chronic obstructive pulmonary (COPD), there is a trial (Phase II) sponsored by Osiris Therapeutics (ClinicalTrials.gov Identifier: NCT00683722) being conducted in order to evaluate the safety and efficacy of *ex vivo* cultured adult human MSCs obtained from bone marrow of healthy adult donors (PROCHYMAL<sup>®</sup>) when administered intravenously to subjects with moderate to severe COPD. PROCHYMAL<sup>®</sup> is also being studied in Phase II trials for the treatment of other conditions such as Type I diabetes and acute myocardial infarction. In addition, the Botucatu Unesp from Brazil is developing a COPD trial focused mainly on patients with a diagnosis of advanced stage pulmonary emphysema (Stage IV dyspnea) (ClinicalTrials.gov Identifier: NCT01110252). In this study, the researchers stimulate the production of granulocytes before the procedure; then MNCs are isolated and afterwards infused through brachial vein.

### Pulmonary Hipertension

It exists two approaches in order to treat pulmonary hypertension (PAH), one using transfected cells and another using autologous endothelial progenitor cells. In 2005 a preclinical study examining the efficacy of bone marrow-derived EPCs in an experimental model,<sup>39</sup> it was found that only animals receiving EPCs transduced with human endothelial NO-synthase (heNOS), exhibited a significant reversal of the established disease. These data are the basis for a Phase I clinical trial (ClinicalTrials.gov Identifier: NCT00469027) sponsored by Northern Therapeutics and conducted in Canada with 18 patients. This study seeks to establish the safety of pulmonary artery injection of genetically engineered autologous progenitor cells transfected with heNOS in patients with PAH in whose have failed all conventional treatments. Once in the lung, these cells are efficiently filtered and engrafted at the level of the smallest arteries. From this location, they are able to release therapeutic gene products, which can then act on the pulmonary microcirculation, as well



as participating directly in the regeneration of the damaged lung vessels. Trial data is expected to become available at the end of 2010.

Based in positive results obtained in dogs another study was performed by Takahashi M et al<sup>40</sup> (ClinicalTrials.gov Identifier: NCT00641836) using an autologous EPCs injection in patients with Idiopathic Pulmonary Arterial Hypertension (IPAH) in order to establish the ideal dose, the duration of therapeutic effect, and potential toxicity. This trial was completed on 2007 and its results were published, showing that intravenous infusion of autologous EPCs seemed to be feasible and safe, and might have beneficial effects on exercise capacity and pulmonary hemodynamics in IPAH patients.<sup>41</sup>

## **Lung Cancer**

In this field, most studies have mainly focused on small cell cancer (ClinicalTrials.gov Identifier: NCT00003860 and NCT00011921). The majority of these approaches combines chemotherapy with stem cells or drugs and are Phase II or III studies, respectively. There are also some trials for the treatment of other kind of tumours such as metastatic solid tumours or metastatic cancer; using, in the first case, peripheral stem cells and white blood cell transfusion (ClinicalTrials.gov Identifier: NCT00003839) and in the second case, treating patients with chemotherapy combined with peripheral stem cell transplantation (ClinicalTrials.gov Identifier: NCT00003943). While this last study has been completed the results are still not published.

## **STEM CELLS AND LIVER DISEASES**

The liver has a remarkable regenerative capacity, which can occur through three different cell lines: Hepatocytes, intrahepatic stem cells and extrahepatic stem cells.<sup>42</sup> Already, a number of patients have been treated for metabolic disorders by transplantation of mature adult hepatocytes.<sup>43</sup> However, this kind of transplant is limited by the number of cells that need to be expanded and transplanted to achieve clinical significance along with the availability of adequate liver tissue and other circumstances. Intrahepatic progenitor cells (oval cells) are thought to be located in the Canals of Hering of the adult liver.<sup>44</sup> However, during liver development, another stem cell population, the 'side population cells', have been found to contribute to both hematopoietic and epithelial lineages.<sup>45</sup> Furthermore, extrahepatic stem cells like bone marrow stem cells show plasticity due to the fusion of BM-derived cells with host hepatocytes rather than the transdifferentiation of extrahepatic cells.<sup>46</sup> However, it is generally accepted that the number of hepatocytes produced is too low to have any clinical impact at present.<sup>47</sup> Despite prior evidence, a growing number of human clinical studies are investigating the effects of bone marrow stem cell therapy in patients with liver disease since these cells are already being handled and transplanted in clinical practice. Moreover, human adipose tissue-derived MSCs can be differentiated to become hepatocyte-like, with similar gene expression, morphology and metabolic activity.<sup>48</sup> However, the long-term phenotypic stability of the transplanted cells is still unknown. In addition, pluripotent human ESCs can be differentiated towards the hepatic lineage by both spontaneous differentiation via the formation of embryoid bodies and directed differentiation using ESC-derived hepatic endoderm inducing factors.<sup>49</sup> However, as ethical concerns may result in limitations on the use of ESCs, the development of iPSCs may therefore prove useful.



## Cirrhosis

Currently, the majority of clinical trials concerning liver tissue are focused on hepatic cirrhosis using different kind of cells like CD133<sup>+</sup>, MSCs or CD34<sup>+</sup>. There are two finished trials from Iranian groups evaluating the injection of stem cells in portal vein. One of them used hepatocyte progenitors derived from autologous MSCs and the other used BM-MNCs enriched with CD133<sup>+</sup> cells. Data showed that MSCs injection can be used for the treatment of end-stage liver disease. Furthermore, this treatment may also improve clinical indices of liver function.<sup>50</sup> The Federal University of Rio de Janeiro conducted a trial (ClinicalTrials.gov Identifier: NCT00382278) to evaluate the kinetics of cellular therapy using BM-MNCs, labelled with 99 mTc and infused through the hepatic artery. However, this study was terminated when other authors showed the same metabolic effect using peripherally injected cells with a lower risk and cost than hepatic artery injection. Studies currently recruiting participants include a Phase I trial at the University of Bologna (ClinicalTrials.gov Identifier: NCT01025622) that uses autologous CD133<sup>+</sup> stem cells administered through the hepatic artery to test its feasibility in cirrhotic patients in an end-stage disease or a Phase II trial developed for Sun Yat-sen University (ClinicalTrials.gov Identifier: NCT 009762867) to determine the efficacy of autologous BM-MSCs transplantation via hepatic artery compared to conservative therapy.

## Chronic Liver Disease

One of the first clinical studies using stem cell therapy for patients with chronic liver disease was published in 2007.<sup>51</sup> Thirty patients on the liver transplant waiting list received BM cell therapy or placebo in a nonblinded study. Patients who received treatment showed an increase in albumin level and a decrease in their Child-Pugh score at the conclusion of the study compared with controls. In January 2010, the same group published the results of a study testing infusion of BM-MNCs in chronic liver disease with a one-year follow-up. They conclude that improvement appears in the first 90 days after the infusion and proposed to either combine cell therapy with granulocyte colony stimulating factor (G-CSF) or perform an additional infusion to repeat the infusion to improve results.<sup>52</sup> Smaller observational clinical studies have illustrated the safety and feasibility of G-CSF administration followed by leukapheresis and re-infusion of CD34<sup>+</sup> cells in patients with chronic liver disease, reporting that the patients showed improvement in clinical status and biochemistry.<sup>53,54</sup> Phase I trials employing a bioartificial liver device have already shown some promise with patients exhibiting improvements in both neurological state and haemodynamics, but this procedure is currently limited by the use of xenogeneic materials. However, these devices permit some restoration of liver metabolic function and in the future are likely to act as a bridge to liver transplantation in patients with advanced or end-stage liver failure.<sup>55,56</sup> Currently, the main constraints on the possibilities of cellular transplantation remain to be difficulties in sourcing and maintaining viable hepatocytes.

Moreover, there are currently several active or completed trials on using stem cell therapy to treat liver insufficiency, hepatectomy and thalassemia. In the case of liver insufficiency, a study conducted by the Imperial College (London) used either hepatic artery or portal vein infusion of adult CD34<sup>+</sup> stem cells. Three of the 5 participants showed improvements in serum bilirubin and 4 of 5 showed improvements in serum

albumin. The Anderson Cancer Center (Texas) has also recently completed a study to determine if the CD34+ stem cells administered by hepatic artery infusion produce liver stem cells following the transplant. They also sought to evaluate the safety of this procedure in patients with severe hepatic dysfunction (ClinicalTrials.gov Identifier: NCT00062543). Finally, the Seoul National University Hospital is recruiting patients requiring an extended hepatectomy for a trial to evaluate the efficacy of treating them using autologous peripheral stem cells to facilitate liver regeneration and evaluate liver function and volume (ClinicalTrials.gov Identifier: NCT01108380). One group of patients will be treated with autologous CD34+ cells, the other group with MNCs and the control group will just suffer the portal vein embolization. All the groups will receive an injection of G-CSF prior the stem cell harvest. With the results of this study scheduled to be collected during December 2011.

## STEM CELLS AND HEART DISEASES

The biggest clinical challenge in this field is to develop new strategies to enhance the regeneration of functional cardiac muscle in the failing heart. Recent studies have suggested that some degree of cardiomyocyte regeneration may occur even in mammals<sup>57,58</sup> although this capacity is very limited.<sup>59,60</sup> Beltrami suggested that the cardiomyocytes lost in a heart attack are replaced in 18 days while the entire set of myocytes in the heart is replaced every 4.5 years. Moreover, several undifferentiated cell populations have been isolated from neonatal and acutely infarcted failing hearts. The number of these cells is increased after Acute Myocardial Infarction (AMI), and is very low in failing hearts, suggesting that these cells take part in ongoing repair, which becomes insufficient in Heart Failure (HF).<sup>57</sup>

Bone marrow, skeletal muscle, fat, cardiac muscle, and umbilical cord blood have all been used as sources of uncommitted progenitor cells for cardiovascular stem cell therapy.<sup>61</sup> But the most promising and challenging of these sources is likely those from the adult heart. When Messina et al<sup>62</sup> developed methods to derive myogenic cells from adult cardiac biopsies they opened a new and interesting area of research aimed at deriving, isolating and expanding cardiac stem cells. Ideally, this cell population would survive in the injured myocardium, giving rise to mature cardiomyocytes and vasculature that integrate with the surrounding host tissue. Data suggest that this is feasible, although it appears to be a rare event.<sup>63</sup> Bone marrow is the most commonly used cell source for cardiac repair, since the ability of BM-MNCs to differentiate into cardiomyocyte-like cells has already been established.<sup>64</sup> In fact, more than 1.000 patients have been treated, usually following AMI, in Phase I and II clinical studies with bone marrow aspirate containing a cocktail of stem progenitor cells. Recent analysis of the obtained results suggest that BM-derived cells provide a significant, but small, benefit correlated with increased myocardial perfusion when administered post-AMI without leading to serious adverse events.<sup>65-67</sup> However, although a reduction in infarct size was observed, there was no significant functional improvement.<sup>68</sup> In an experimental model of BM-treatment of infarction what was first thought to be the formation of new cardiomyocytes, was later found to be the result of transdifferentiation and cell fusion as was subsequently demonstrated.<sup>69-71</sup> It has also been proposed that the observed improvement in left ventricular ejection fraction was most likely due to a paracrine effect however, it appears while that positive

inotropic effects do not always translate into improved survival in the post-infarcted heart and moreover it also remains to optimize engraftment once the cells have been delivered to the heart.<sup>72-74</sup>

Skeletal muscle-derived myoblasts (SKMBs) are considered a good option for cardiac cell therapy due to different reasons: They can be easily obtained from the same patient avoiding immunosuppression, rapidly expanded in vitro and then transplanted in the patient's heart. Unfortunately, myoblasts have not been able to differentiate into cardiomyocytes and they do not integrate electrically with the host cells. Despite this evident problem, several nonrandomized clinical trials have been performed to evaluate the possibility of treating infarcted heart tissue with myoblasts. These trials demonstrated a thickening of the left ventricles, an increase in ejection fraction and prevention of left ventricular dilatation with clinical improvement seen in some patients. The clinical procedure was well tolerated but resulted in a high incidence of arrhythmias, sometimes with fatal consequences. Unfortunately, human trials with myoblasts have yielded disappointing results which may be due to the following circumstances: First, it was found that at least 75% of the transplanted cells died within the first few days; second, the transplanted myoblasts remained localized around the injection site, rather than homing to sites of injury lastly, without appropriate immunosuppression, any surviving myoblasts were rapidly rejected within two weeks.<sup>31,75-78</sup>

Concerning EPCs, following intravenous infusion of these cells into an infarcted region, researchers found a marked increase in capillary density within the infarcted area and its borders.<sup>79</sup> This effect has been attributed to a combination of vasculogenesis and angiogenesis, and several paracrine properties that have recently been attributed to these cells.<sup>80</sup>

A new and promising approach in terms of cardiac tissue regeneration is the creation of an autologous-cell-derived bioartificial heart that could solve the two major problems associated with heart transplantation: The shortage of donor organs, and the side effects of life-long immunosuppression. Recently, the group of Doris A. Taylor from the University of Minnesota developed a perfusion method to remove all cellular constituents from cadaveric heart tissue to obtain a three-dimensional scaffold comprising native cardiac extracellular matrix that retains the original four chambered geometry and architecture of the native heart subsequently the remaining extracellular matrix is then relined with functional endothelial cells.<sup>81</sup> The recellularization of this 'scaffold' was performed by the infection of neonatal cells into the matrix while housing it in a bioreactor. The construct matured over time, and by days 8-10, they found observable contractions of the recellularized left ventricular segments. Histological examination of the recellularized construct showed live cells expressing myosin heavy chain protein, connexin 43, von Willebrandt factor and other markers. The same research group also showed contraction using pluripotent human stem cells on a decellularized cardiac matrix. Until now they have no attempted a human organ. The cells that could give rise to vasculature would be derived from autologous bone marrow or peripheral blood mononuclear cells known to participate in angiogenesis. However, to build cardiac muscle and valves, the hypothesis would have to work on cardiac derived stem cells or human iPSCs.<sup>82</sup> In this context, D.A. Taylor is also developing an ambitious project, in order to construct several human bioartificial organs, such as liver, in collaboration with Dr. Fernández Aviles from Hospital Gregorio Marañón in Madrid (Spain) and the Spanish Transplant Organisation (ONT). However, several years will be needed to test all these principles.

## Infarction

Besides the studies performed in AMI, the REPAIR-AMI trial was the first randomized Phase II study showing that BM-MNCs participate in tissue repair. However, a sufficient degree of tissue injury was found to be required for the cells to show efficacy.<sup>83</sup> Following the REPAIR-AMI trial the group of Andreas M. Zeiher planed another study named REPAIR-ACS that will extend the above findings to patients with non-ST segment elevation myocardial infarction. A study which results have been recently published (June 2010) is the TECAM trial that evaluates a putative negative effect on coronary atherosclerosis in patients who receive an intracoronary infusion of unfractionated BM-MNCs following an acute ST-elevation myocardial infarction. They found that intracoronary injection was not associated with accelerated atherosclerosis progression at mid term. Currently, there are many other studies related to infarct that are ongoing or recruiting participants, a list of which is seen in Table 2.

## Heart Failure

In the case of heart failure, data on approximately 250 patients that received SKMBs have been published. It demonstrated a relationship between contractile impairment at baseline and the left ventricular functional improvement following treatment with SKMBs, due to the fact that the patients with severe impairment of myocardial function may not experience extensive benefits compared to patients with lesser myocardial damage. The MAGIC trial, the first randomized placebo-controlled study on SKMBs was published in 2008.<sup>84</sup> This was an international study of 97 heart failure patients with left ventricular ejection fraction ranging from 15 to 35% that compared the safety and efficacy of both low ( $400 \times 10^6$ ) and high ( $800 \times 10^6$ ) doses of autologous expanded SKMBs over 3 weeks vs. placebo. The cells were delivered into the myocardium following bypass grafting via injection. The high dose SKMBs group did exhibit a significant reduction of left ventricular end-systolic and end-diastolic volumes vs. placebo which supports a role for using myoblasts in remodelling heart muscle. However, results of this study showed that injections of SKMBs did not improve either global or regional left ventricular function as compared to placebo. There was also no significant difference in the incidence of arrhythmias between groups. The limitations of this trial include the small number of patients and the relatively short length follow-up.

In other trials like CAuSMIC ischemic cardiomyopathy patients were subjected to SKMBs administration concurrently with CABG (Coronary artery bypass graft) as an adjunct to a left ventricular device implanted as a bridge to transplantation.<sup>85</sup> Following procedure, there were improvements in myocardial perfusion and left ventricular ejection fraction increased. Later, several explanted hearts were examined and engrafted SKMBs were found in almost all the specimens within the infarcted regions. Furthermore, in the SEISMIC trial myoblasts were infused via an endoventricular needle-injection catheter in congestive heart failure patients previously fitted with implanted cardiac defibrillators (ICDs), receiving standard medical therapy and who are experiencing congestive heart failures. The results demonstrated both safety and some clinical improvement. On September 2009, Bioheart, Inc. presented positive efficacy data from the SEISMIC trial and also introduced the MARVEL Clinical Program, a Phase II/III clinical trial designed to assess any changes in functional capacity and quality of life, in patients with advanced heart failure after receiving injections of adult muscle

stem cell therapy into damaged heart muscle. This trial is the largest study of its kind to utilize catheter-based cell delivery in a double-blinded, placebo-controlled manner. These patients, who suffered chronic HF and many of whom were also diabetic, were randomly assigned to three separate treatment groups depending on the number of cells administered directly into parts of the ventricular wall specifically weakened by scar tissue from previous heart attacks. The results presented were based on analyses of 3 and 6-month follow-up data from 20 patients. Over the 6-month observation period, pronounced changes were seen in the cell-treated groups comparing to controls, suggesting that patients with heart failure could return to a more active lifestyle after receiving this treatment. The occurrence of early (within 4 weeks of cell implantation) ventricular tachyarrhythmia appeared in the airway smooth muscle cells of treated groups, which is similar to observations in other clinical trials involving heart failure patients. However, these arrhythmias were detected and treated with no adverse results and disappeared early on in many cases while completely disappearing by six months posttreatment. The investigators believe that early detection and management strategies, as were implemented and refined during enrolment for MARVEL Part I, reduced the risk of recurrent arrhythmias and may enhance 6-month event-free survival. Furthermore, there were no deaths reported in this study.

Recently published studies examining patients with a mean baseline left ventricular ejection fraction of 30% or lower found symptomatic benefits after the SKMBs procedure. It is possible that this degree of functional improvement might not only depend on baseline left ventricular ejection fraction, but also on the route of delivery. Moreover, CABG could increase blood flow and augment tissue perfusion in order to improve engraftment so that a larger proportion of cells can contribute to repair.

The use of BM-MNCs has recently been investigated in HF patients. These studies, TOPCARE-HF and BOOST-II, were initiated to gain a more systematic insight into the response of the myocardium to direct injection of BM-MNCs during HF pathophysiology. These trials showed that a small number of patients undergoing targeted placement of BM-MNCs in ischaemic zones could be delisted from transplantation because of increased exercise tolerance. Unlike the SKMBs trials, symptomatic and functional improvements in HF patients treated with BM-MNCs occurred without adverse electrical events. In TOPCARE the conclusion was that the intracoronary infusion of progenitor cells is safe and feasible in patients with healed myocardial infarction. Furthermore, the transplantation of bone marrow cells (BMCs) is associated with a moderate but significant improvement in left ventricular ejection fraction after 3 months. BOOST II was a randomised, double-blind trial that studied the effects of using either 85%-90% or 91%-95% arterial oxygen saturation. Researchers concluded that intracoronary transfer of autologous BMCs promotes improvement of left ventricular systolic function in patients who have suffered an acute myocardial infarction. Other trials currently recruiting participants are shown in Table 3.

To summarize; until the present, the trials performed have focused on the use of BM-MNCs, EPCs, MSCs, and cardiac-derived progenitor cells (CPCs) as treatment for a broad range of cardiovascular diseases, from advanced coronary atherosclerosis to end-stage HF. Unfortunately, the outcomes of these studies are quite divergent due to differences in disease context, patient population, cell type, dose and some other factors. But, data on the use of BM-MNCs in advanced atherosclerotic disease, AMI and ST-elevation myocardial infarction (STEMI) are encouraging. The improvement in symptomatology following treatment with BM-MNCs was found to correlate with

**Table 2.** Current clinical trials in the field of infarct. Most of them are still recruiting participants.

Name	Identifier	Type of Cells	Delivery	Participants	Type of Study	Institution
REPAIR-ACS	NCT00711542	Autologous BM-derived progenitor cells	Intracoronary infusion	Coronary artery disease after Non-ST segment elevation myocardial infarction	Phase I/II	Johann Wolfgang Goethe University Hospitals
PROMETHEUS	NCT00587990	Autologous hMSCs	Intramyocardial injections following CABG surgery	CABG in Left ventricular dysfunction secondary to myocardial infarction	Phase I/II	NHLBI and Johns Hopkins University Specialized Center for Cell Based Therapy
PERFECT	NCT00950274	CD133+ autologous BM stem cells	Intramyocardial injections after CABG surgery	CABG in Coronary artery disease as consequence of infarct	Phase III	Miltenyi Biotec GmbH
REGEN-AMI	NCT00765453	Autologous BM-derived progenitor cells	Intracoronary injections through percutaneous route	AMI	Phase II/III	Barts and The London NHS Trust
TIME	NCT00684021	Autologous BM-MNCs	Infusion through catheter into the damaged area	Recent Heart Attack	Phase II	NHLBI
Late TIME	NCT00684060	Autologous BM-MNCs	Infusion through catheter into the damaged area	Heart attack 2 to 3 weeks before	Phase II	NHLBI

*continued on next page*

**Table 2.** Continued

Name	Identifier	Type of Cells	Delivery	Participants	Type of Study	Institution
POSEIDON	NCT01087996	Auto-hMSCs Allo-hMSCs	Transendocardial injections during cardiac catheterization	Chronic left ventricular dysfunction secondary to myocardial infarction	Phase I/II	NHLBI and University of Miami
REVITALIZE	NCT00874354	Autologous BM-derived mononuclear cells	Intracoronary application through infarct artery	AMI	Phase I	Cedars-Sinai Medical Center
MESENDO	NCT00548613	Two types of bone marrow-derived progenitor cells	Intracoronary or intramyocardial infusion	AMI AMI + CABG	Phase I	TCA Cellular Therapy
CADUCEUS	NCT00893360	Autologous Cardiosphere-derived stem cells	Intracoronary delivery	AMI + Left ventricular dysfunction	Phase I	Cedars-Sinai Medical Center Johns Hopkins Hospital NIH NHLBI The EMMES Corporation



Table 3. Current clinical trials in the field of heart failure (HF)

Name	Identifier	Type of Cells	Delivery	Participants	Type of Study	Institution
ALCADIA	NCT00981006	Autologous hCSC	Intramyocardial injection	Severe refractory heart failure patients with chronic ischemic cardiomyopathy	Phase I	Prefectural University of Medicine
	NCT00418418	Autologous BM-derived MSCs	Intramyocardial transplantation	Patients with systolic heart failure, with ischemic coronary heart disease, scheduled to by-pass-operation (CABG)	Phase II	University of Helsinki
IMPACT-CABG	NCT01033617	Autologous BM-CD133+	Intramyocardial injections at the time of surgery	Patients undergoing CABG with left ventricular dysfunction	Phase II	Centre hospitalier de l'Université de Montréal
REGEN-IHD	NCT00747708	Autologous BM-derived stem cells	Percutaneous intracoronary or intramyocardial injection	Patients with heart failure secondary to ischaemic heart disease	Phase II/III	Barts and The London NHS Trust
FOCUS	NCT00824005	Autologous BM-MNCs	Intramyocardial injection under electromechanical guidance	Patients with Chronic ischemic heart disease and left ventricular dysfunction	Phase II	NHLBI and CCTRN
	NCT00721045	Allogeneic mesenchymal precursor cells (MPCs)	Transendocardial injection	Heart failure	Phase II	Angioblast Systems
	NCT00620048	Autologous CD34+ cells	Intramyocardial injections	Congestive heart failure	Phase I	D. Losordo
SCIPIO	NCT00474461	Cardiac stem cells (CSCs)	Intracoronary injection	Patients with ischemic cardiomyopathy	Phase I	University of Louisville

increased myocardial perfusion. Unfortunately, BM-MNCs given to treat reperfused and/or stented AMI were not as beneficial. Although a reduction in the size of the infarcted area was observed, no functional improvement was gained. This may be because unfractionated BM-MNCs, contain both mature and immature EPCs along with other progenitors, and it is quite possible that a combination of these cells may be the best choice, although specific CPCs populations have not yet been clinically tested until this moment. However, currently there are also several trials recruiting participants to test the use of CPCs, such as the SCIPIO trial (Table 3) in which stem cells are harvested from right atrial appendages and injected intracoronary to test the improvement in contractile function and general clinical status.

## STEM CELLS AND KIDNEY DISEASES

The most studied stem cells for cellular therapy of kidney diseases are MSCs and HSCs along with unfractionated bone marrow. These cells are able to repair injured kidneys by either replacing damaged cells or by improving regeneration from surviving, mature cells. The latter mechanism is modulated via paracrine delivery of small molecules such as cytokines to the site of regeneration. A Phase II multicenter randomized, controlled, open-label trial compared the survival advantage of using a renal tubule assist device (RAD) composed of a conventional hemofilter lined by monolayers of renal cells compared to continuous renal replacement therapy (CRRT) in 58 patients with acute kidney injury (AKI).<sup>86</sup> This study found there was no survival advantage observed of RAD over CRRT at 28 days, it reached statistical significance at 180 days. The results of this initial study of RAD in AKI are encouraging. There is also an ongoing nonrandomized open label Phase I clinical trial evaluating safety of an intra-aortic infusion of human MSCs in adult patients at high risk of developing AKI following cardiac surgery (ClinicalTrials.gov Identifier: NCT00733876). The results on this study will be critical for expanding the use of MSCs therapy in patients with AKI.

## STEM CELLS AND OTHER TISSUES

### Diabetes

There have been several trials evaluating stem cell therapy for both Type 1 and Type 2 diabetes mellitus using different kind of cells, including MSCs, autologous BM-MNCs or CD34<sup>+</sup> cells, injected into either the gastroduodenal artery or the tail of the pancreas. Moreover, a successful clinical trial involving autologous HSCs transplantation and immunosuppression in diabetic patients was recently reported.<sup>87</sup> This trial was a Phase I/II study of 15 patients with newly diagnosed Type 1 diabetes, with the stem cells being injected intravenously. Both high-dose immunosuppression and HSCs infusion was performed with acceptable toxicity, with the only adverse effect observed being culture-negative bilateral pneumonia in one patient and late endocrine dysfunction (hypothyroidism or hypogonadism) in 2 others. Beta cell function was increased in all but one patient, and induced prolonged insulin independence was seen in the majority of the participants. However, before reaching any conclusions on the use of stem cells in diabetes mellitus therapy, it will be necessary to wait for further results.

### Chronic Critical Limb Ischemia

Critical limb ischemia (CLI) is defined as rest pain or tissue necrosis with ulceration or gangrene, and is generally the result of peripheral arterial occlusive disease, possibly leading to amputation of the affected limb with diabetic patients having the highest risk of amputation.<sup>88,89</sup> However, a large number of patients are not eligible for revascularization procedures due to anatomic location of the lesions or the extent of disease.<sup>90</sup> Furthermore, there is no currently available effective pharmacologic therapy.<sup>91</sup> Consequently, exploring new strategies for revascularization of ischemic limbs is of major importance. While, BM-derived progenitor cells have been identified as a potential new therapeutic target for treating CLI, the majority of studies examining the efficacy of progenitor cell therapy for CLI have used intramuscular implantation method of whole MNCs fractions.

The Therapeutic Angiogenesis by Cell Transplantation study (TACT), the first clinical pilot trial for therapeutic angiogenesis using BM-MNCs in patients with chronic limb ischemia was published in 2002 by a Japanese group. This study reported a significant improvement in transcutaneous oxygen pressure, rest pain, ankle brachial index and pain-free walking time in patients with critical limb ischemia following intramuscular implantation of autologous BM-MNCs. The second part of the TACT study was a randomized controlled trial of 22 patients with bilateral limb ischemia who randomly received implantation of BM-MNCs in one leg and peripheral blood derived MNCs as a control treatment in the other. Improvements were seen in lesion size within the BM-MNCs injected legs showing much smaller increases than controls. The authors suggested that the efficacy of the treatment was correlated to the CD34<sup>+</sup> content and angiogenic factors. In 2008 the same group published the results of a multiinstitutional follow-up study in 115 patients to establish the long-term safety and clinical outcome of intramuscular BM-MNCs implantation to ischemic limbs. They found that this therapy promoted long-term improvement in limb ischemia, leading to an extension of the amputation-free interval. The safety and efficacy were comparable to conventional revascularization therapies.<sup>92</sup> As follow-up to the studies on the potential of CD34<sup>+</sup> cells, a Phase I/II trial of transplantation of autologous CD34<sup>+</sup> cells was performed in no-option patients with atherosclerotic peripheral artery disease or Buerger's disease with critical limb ischemia (CLI). During the 12-week observation period following cell therapy, the study found that the Wong-Baker FACES pain rating scale, TBPI, transcutaneous partial oxygen pressure, total or pain-free walking distance, and ulcer size serially improved in all patients. Furthermore, no death or major amputation occurred, and severe adverse events were rare.<sup>93</sup> Another finished trial is the OPTIPEC study that also demonstrated the increased angiogenesis after a local injection of BM-MNCs.<sup>94</sup> Currently, among the studies that are ongoing at this moment we could find the JUVENTAS (ClinicalTrials.gov Identifier: NCT00371371) developed by the Universitair Medisch Centrum Utrecht that performed intra-arterial infusion of autologous BM-MNCs into the common femoral artery.

Thus, progenitor cell-based therapy for CLI has been shown to be safe, feasible and to effectively promote neovascularisation in ischemic tissue, leading to improvement in clinical outcomes. Developments in this treatment may lead to limb salvage, ulcer healing, and CLI down staging, with major consequences for quality of life.

### **Crohn's Disease**

Rectovaginal fistulas in patients with Crohn's disease are difficult to solve with surgical treatment having a high failure rate. Taking into account that MSCs extracted from certain tissues, such as adipose tissue, can differentiate into various types of cells;<sup>95</sup> García-Olmo published in 2003 the results obtained in a young patient whose fistula was treated with autologous stem cells isolated from a lipoaspirate.<sup>96</sup> This positive finding was the basis for a Phase I trial on the treatment of Crohn's fistula using adipose derived MSCs transplantation.<sup>97</sup> Five patients with a variable number of fistulas participated in the study and no immediate adverse reactions were observed in any of the cases. Follow-up examinations showed a complete healing in six of eight procedures. Currently this group is developing the Phase II trial named ALOREVA that uses expanded allogenic adipose-derived adult stem cells.

### **Pressure Ulcers**

Pressure ulcers are the result of ischaemic damage to soft tissues induced by unrelieved pressure over a bony prominence and are a major health problem for bedridden patients or persons with reduced mobility. Among these patients, spinal cord-injured (SCI) subjects are especially prone to developing this kind of lesion. About 85% of SCI patients develop pressure ulcers during their lifetime and in 8%, pressure sores are the cause of death.<sup>98</sup> Taking into account that recent studies have identified the role of BM cells in host defence and inflammatory processes in the skin, including wound healing and given that treatment strategies for pressure ulcers can be both costly and complex,<sup>99-101</sup> Our research group examined the possibility of improving pressure ulcer treatment by using autologous BM-MNCs obtained by anterioposterior iliac crest aspiration.

Cell infusion was performed in the operating room using an aseptic technique. First, the plastic surgeon washed the bursa repeatedly with saline solution to eliminate all traces of topical treatment. Without removing the bursa, the ulcer margins were revitalized and the wound was closed to create a pouch into which the cell suspension was injected using a catheter. Full healing was observed in 11 of the 12 ulcers which translates to a cure rate of 91.67%. NMR and CT imaging confirmed the complete disappearance of the ulcer in the 11 patients. Actually, a larger study is being prepared in order to confirm these preliminary results.

### **CONCLUSION**

Progenitor cells represent a potential new therapeutic target for which several clinical studies have shown promising results. However, larger, randomized, blinded, placebo-controlled trials are needed to provide definitive proof of the clinical effects of progenitor cell therapy in these patients, and to evaluate the best form of therapy for specific patient groups. In addition, questions regarding the cell population to be used, optimal dose and routes of delivery will have to be addressed. It is hoped that in the coming years better insight into the mechanisms underlying the beneficial effects of progenitor cell therapy will help the design of more specific targeted therapies.

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## CHAPTER 20

# TISSUE BIOENGINEERING AND ARTIFICIAL ORGANS

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**Abstract:** The scarcity of organs and tissues for transplant and the need of immunosuppressive drugs to avoid rejection constitute two reasons that justify organ and tissue production in the laboratory. Tissue engineering based tissues (TE) could allow to regenerate the whole organ from a fragment or even to produce several organs from an organ donor for grafting purposes. TE is based in: (1) the ex vivo expansion of cells, (2) the seeding of these expanded cells in tridimensional structures that mimic physiological conditions and, (3) grafting the prototype. In order to graft big structures it is necessary that the organ or tissue produced “ex vivo” bears a vascular tree to ensure the nutrition of its deep layers. At present, no technology has been developed to provide this vascular tree to TE derived products. Thus, these tissues must be thin enough to acquire nutrients during the first days by diffusion from surrounding tissues. This fact constitutes nowadays the greatest limitation of technologies for organ development in the laboratory.

In this chapter, all these problems and their possible solutions are commented. Also, the present status of TE techniques in the regeneration of different organ systems is reviewed.

## INTRODUCTION

During recent years, organs and tissue transplantation have increased in most developed countries. However, there are four limiting factors restricting the use of organs and tissues from donors. The most critical of these limitations is the shortage of organs suitable to be transplanted. The second is the need to use immunosuppressive

medications to prevent rejection, which tend to have substantial side effects. In addition, not every organ is suitable to be transplanted. For example, it is not possible to transplant components of the nervous system. Finally, transplanted organs have a limited lifetime; the average life expectancy of a transplanted heart is around 10 years.<sup>1</sup> Most of these problems can potentially be solved by the production of new organs and tissues in the laboratory using tissue engineering (TE) techniques.

The purpose of TE is to repair, replace, preserve or improve the function of an organ or tissue.<sup>2</sup> Tissue Engineering is a multidisciplinary area of science that puts into practice principles from both engineering and biology to produce organs and tissues in the laboratory which are able to replace the functions of a damaged organ.

This new technology is based on three main types of structure: Cells, scaffolds and a suitable culture environment (the conditions under which cells are kept). Simplifying, organs and tissues are made up of cells fixed to a three dimensional structure (extracellular matrix). The tissue as a whole (cells and extracellular matrix) is vascularised (providing nutrition, oxygen supply and waste removal) and innervated. In short, every TE strategy requires management of cells and the development of extracellular matrices that not only provide support to these cells but also allows the new tissue to be integrated with that of the recipient, encouraging the generation of new blood vessels and thereby its innervation (Fig. 1).

It is important to point out that currently tissue engineering only offers the possibility of recovering lost function, given that the formation of entire organs and tissues similar to the natural ones is still in the realms of science fiction.

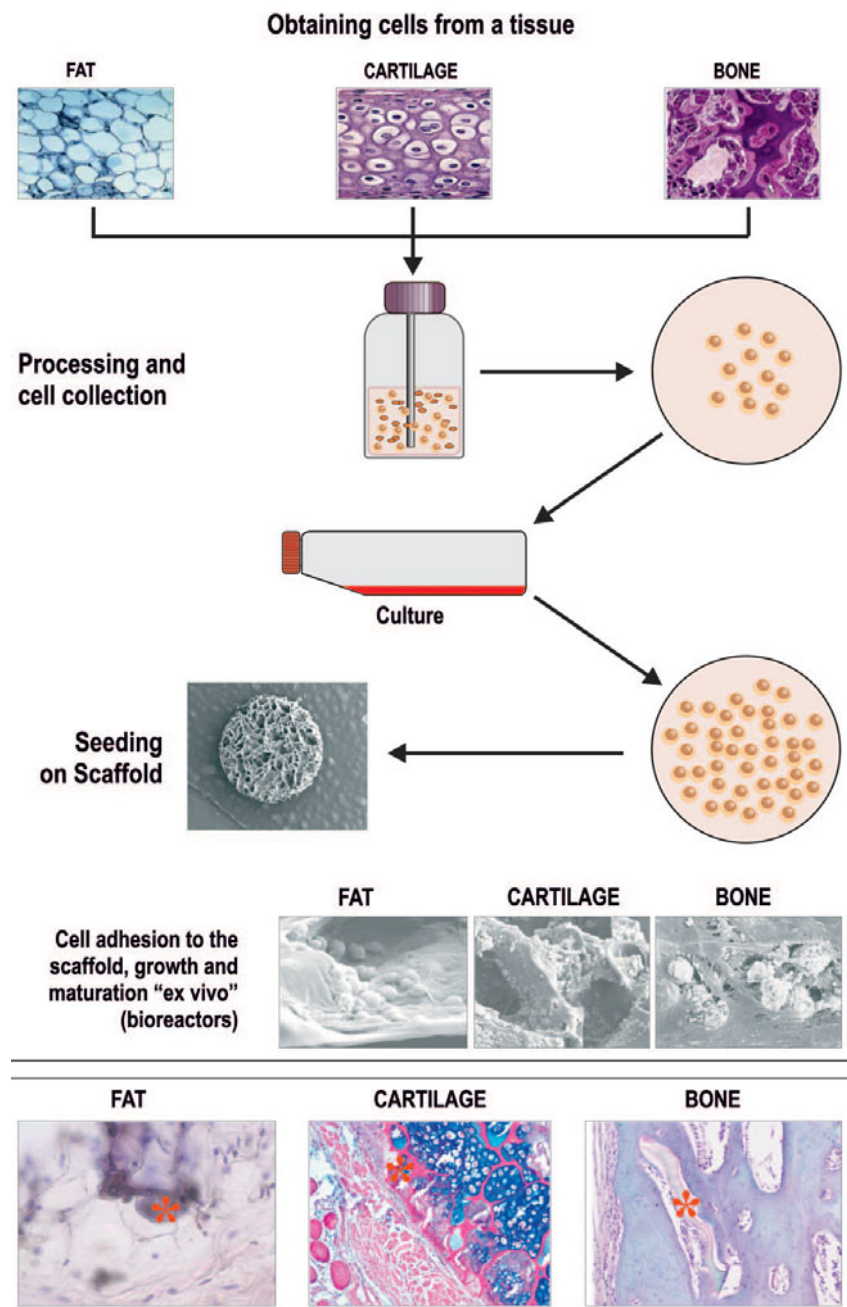
## CELLS

The living and functional part of tissues and organs is composed of cells. It has long since been known that these cells can be managed and kept alive outside the organism. The boom of cell culture techniques took place during the nineteen-forties and their use was generalized during the sixties. The idea of using cell cultures in order to regenerate organs and tissues emerged later. The first type of tissue produced by Tissue Engineering, skin, was not used in clinical practice until 1981.<sup>3</sup> Indeed, the term “Tissue Engineering” was first coined in 1987 during a meeting of the National Sciences Foundation in Keystone, Colorado.<sup>4</sup> Most TE protocols are based on a small biopsy of healthy tissue. For this reason, the initial number of cells is limited and a period of cellular expansion is required. This is carried out using cell culture techniques.

Ideally, the source of cells for use in TE must be easily accessible, and, in addition, cells must have a high proliferative capacity, as well as being able to differentiate into the required cell type, in cases where the source used is different from the damaged tissue.

Cells can be obtained from a fragment of adult tissue to develop a similar tissue. This is the case in, for example, autologous cultures of keratinocytes<sup>3</sup> and chondrocytes.<sup>5</sup> An advantage of this approach is that immunological rejection and the possible transmission of infectious agents are avoided.

A second source of cells is embryonic stem cells (ESC). These cells are multipotent, so they have the ability to develop into cells derived from all three embryonic layers.



**Figure 1.** This figure shows the method for obtaining and processing cells from a tissue, and the seeding in a scaffold. Prototype transplantation leads to resorption of the scaffold and the generation of tissue similar to the original (\*remnants of scaffold).

However, their clinical application is limited due to ethical dilemmas regarding their use and their tumorigenic capacity.<sup>6</sup>

Another cell source is adult stem cells (ASC). These cells are in charge of maintaining the homeostasis of the tissues they live in. Some of these cells have the ability to activate and differentiate into other cell types in order to regenerate a different tissue. However, the tasks of locating, isolating and growing them in culture still present difficulties.<sup>7</sup> In many organs and tissues mesenchymal stem cells (MSC) offer a good alternative. These cells can be used for the production of multiple tissue types (muscle, bone, cartilage, soft tissues, etc.). Given that they also have immunomodulatory properties, they could be used to decrease the immune response of TE prototypes carried out with donor cells.<sup>8</sup>

Placental tissues are yet another possible source of cells for the development of TE models. The cells can be obtained from: Umbilical cord blood, placental tissues and even from the amniotic fluid.<sup>9</sup> These sources share features with embryonic cells, but with the additional advantage of not forming teratomas when injected in vivo, hence their important therapeutic potential.<sup>10</sup>

Recently a new source of cells has been described: Induced pluripotent stem cells (iPSC). Several authors starting from adult cells, have managed to reprogramme these cells by introducing genes that code for transcription factors related to pluripotency.<sup>11</sup> Using this reprogramming combined with gene therapy techniques, it has been possible to regenerate tissues from cells of patients suffering from various congenital diseases.<sup>12</sup> In the near future, the reprogramming of these cells may be achieved by short-term exposure to transcription factors, either by using non-integrating viral vectors or by creating a suitable environment that directs the cells and prompts them to be reprogrammed. For the moment, the only method for reprogramming that is effective relies on retroviral vectors that integrate into the host genome, but it makes these cells potentially oncogenic and difficult to manage. Nevertheless, in due course, this technology may provide us with an endless source of autologous cells for the development of future TE approaches.

## SCAFFOLD

In vivo, cells are embedded in an extracellular matrix composed of a three dimensional fibrous network (collagen and elastic fibres) with glycoproteins (fibronectin, osteopontine, laminine) and proteoglycans (keratan sulphate, chondroitin sulphate, hyaluronic acid, etc.). Although all extracellular matrices share these components, their organization, shape and physical properties varies considerably from one tissue to another, depending on the chemical composition and the three dimensional organization of the components present. The interaction between cells and the extracellular matrix is dynamic. In physiological conditions, cells produce and reshape this extracellular matrix. At the same time, the extracellular matrix controls the behaviour of the cells. So, the extracellular matrix can be used to guide morphological changes, to organize cells and even to control their differentiation.

Cell culture is usually carried out in two dimensions. For this reason, a structure for the cells (scaffold) is needed to enable them to redistribute in three dimensions and thereby recover their functionality. An ideal scaffold must:<sup>13</sup>

1. Maintain its three dimensional structure to be able to guide the regeneration of new tissue once it has been transplanted.
2. Have suitable nano and micro dimensions to enable both the diffusion of nutrients and growth factors, and the removal of waste from the transplanted cells.
3. Have suitable micro and macro dimensions that can be penetrated by the vessels and surrounding tissues to enable re-organisation of the neo-formed tissue.
4. Have a contact surface and physical-chemical properties that favour cell migration, adherence, proliferation and differentiation.
5. Have a controlled degradation rate that allows the re-organization of the tissue and does not provoke adverse reactions (toxic, immune and inflammatory responses) due to scaffold degradation products.

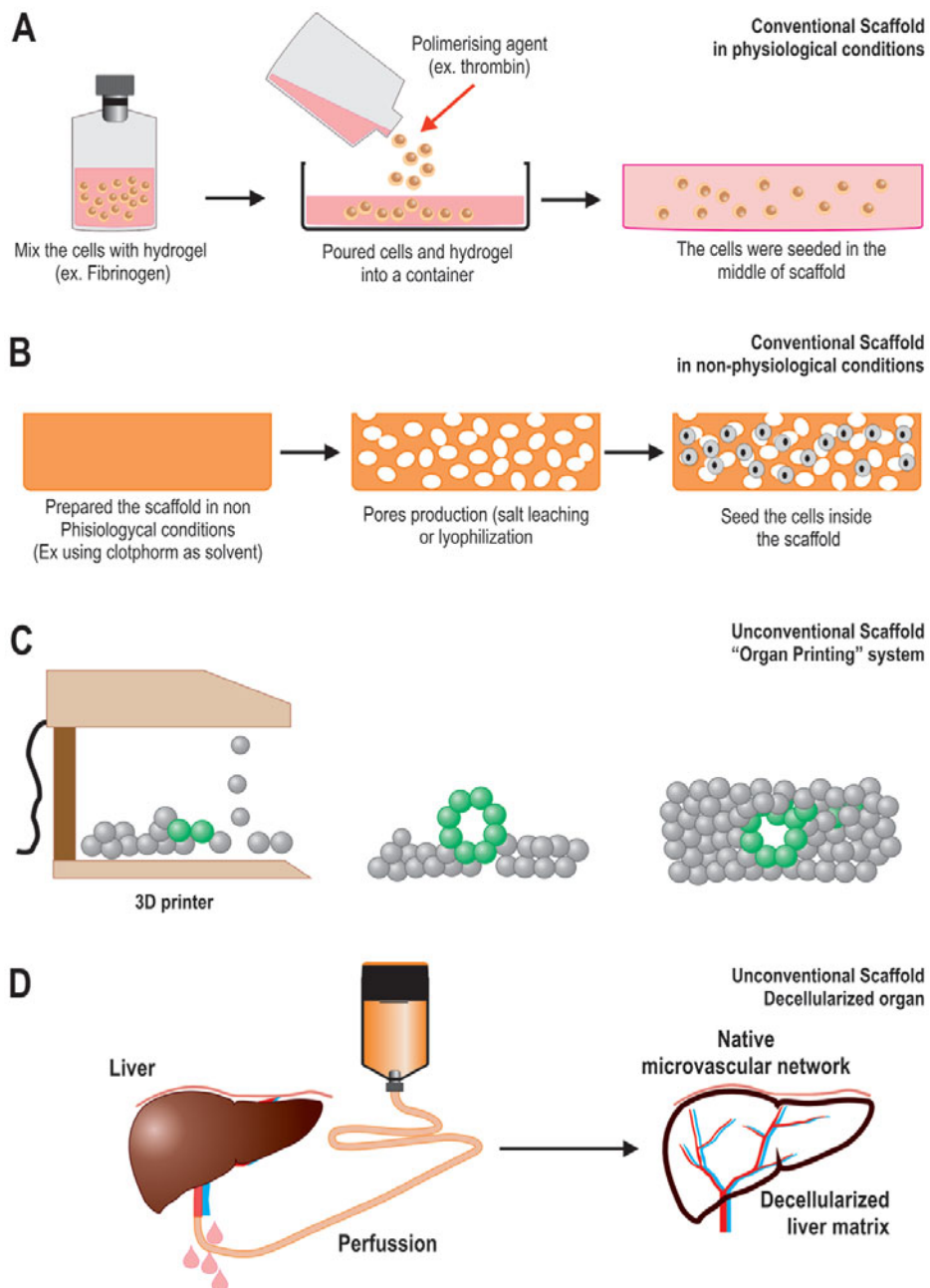
Traditionally, biomaterials that can be used in the synthesis of scaffolds in tissue engineering have been classified into four types: Metals, ceramics, polymers and composites. Metals have a high mechanical resistance but they are not degradable. Ceramic materials have been used in TE of bone tissue because of their mechanical characteristics. However, polymers are the most widely used; this is due to the great flexibility they offer, enabling the composition and structure of the scaffold to be designed depending on the requirements of the tissue.<sup>14</sup> Polymeric materials used in tissue engineering, can be classed as synthetic or natural. Synthetic polymers are those derived from polylactic and polyglycolic acids (PLA, PGA, PLGA), polyethylene glycol (PEG), polyvinyl alcohol (PVA), polyacrylic acid (PAA), etc., while the following are natural polymers: Collagen, fibrin, gelatine, hyaluronate, glycosaminoglycans, chitosan, alginate, dextran, etc. This latter type of polymer scaffold (agarose hydrogels, alginate, and fibrin) can be produced under physiological conditions which means that cells can be embedded during the polymerization process. If polymerization is not performed under physiological conditions, later steps are required to produce pores and then seed the cells onto the scaffold.

With new technologies, an alternative to the classical way of forming the scaffold has become possible, namely, so-called organ printing,<sup>15</sup> which allows the production of more complex scaffolds. In the area of Tissue Engineering, 3D printers combined with image processing software, allow scaffolds to be produced from small fragments. It is possible to direct the assembly of these fragments to form a more complex scaffold, with complete control of the internal structure, including a vascular tree. This latter feature is critical and tends to be the limiting factor in the production of various TE prototypes, as we will see below.

Another important source of scaffolds are native tissues and organs, which have been decellularized, by physical and chemical means. Decellularization enables all the extracellular matrix of a tissue to be recovered, removing the original cells and replacing them by cultured autologous cells. With this technique scaffolds have been obtained from the intestinal mucosa, dermis, tendons and adipose tissue.

Decellularization techniques, first described some years ago, have come back into vogue. One of the advantages of this approach is that the native vascular tree can be used to deliver chemical agents to achieve organ decellularization. With this strategy, it has been possible to obtain organs such as the heart, liver and even lungs<sup>16</sup> with their internal structure intact, including the blood vessels. These scaffolds may be the basis for the future development of complex organs using TE techniques (Fig. 2).





**Figure 2.** Different types of “Scaffolds”. (A) Hydrogel made under physiological conditions. The cells are grown inside easily. (B) Scaffold performed in nonphysiological conditions (Ex. using chloroform as solvent). Required to undergo a process which generates pores (salt leaching or lyophilization). (C) Unconventional Scaffold made with an “Organ Printing” system. It uses a computer controlled design and a 3D printing system. Allows the design of complex structures inside. (D) De-cellularized organ. Keeps the original structure of the organ and the native microvascular network.



## CULTURE ENVIRONMENT

By culture environment we mean the external conditions to which cells that form the tissue synthesized by TE are subjected, prior to the transplant. This environment is determined by the type of culture used, the physical-chemical conditions of the culture system, the growth factors that are added and all the “extras” provided in order that the synthesized tissue is as complete and mature as possible.

### Cell Culture

These external conditions to which cells are exposed to are an essential factor in the development of a tissue through TE. It is necessary to provide cells with a culture environment that allows them to grow and differentiate into the desired-type of cell. The minimal conditions that we should provide are a physiological temperature, isolation from external environment and growth and differentiation factors. These conditions were achieved in conventional cell culture, using incubators set at 37°C, with a 5%-10% CO<sub>2</sub> environment (pH control) and a sterile culture flask (isolation). Cells grew on the base of the culture flask and the growth factors were provided by enriching the culture environment with foetal serum. However, these conditions are not sufficient for the culture and expansion of many types of cell that have more specific requirements. Basic knowledge concerning cell biology accumulated over the last 30 years has made it possible to identify a number of additional growth and differentiation factors that are critical for the ex-vivo development of cells. The development of recombinant DNA technology has allowed commercial synthesis of the peptides that are essential for cell signalling, and today it is easy to obtain these types of products for enriching culture environments.

### Bioreactors

Our organs are constantly receiving mechanical, biochemical, electrical and cellular stimuli. These stimuli help the organ or tissue to function properly. Tissues made using TE should also receive these signals during the ex-vivo phase of culture in order to mature correctly. This can be achieved using bioreactors. Ideally, a bioreactor is a device that needs to be able to provide biological, physiological and mechanical stimuli. Moreover, these stimuli must be applied in a sequential way, both in time and space, in order to provide specific and clear stimulation to the cells and tissues.<sup>17</sup>

Given the different characteristics of each tissue type, there are a variety of bioreactors designs. Simple systems of bioreactors are cultures in continuous perfusion, in which cells grow fed by a culture medium driven by a peristaltic pump. Other commonly used bioreactors include rotary systems, in which cells are subjected to a constant rotation movement, and those in which tissues are subjected to varying pressures, that mimic the systolic and diastolic phases of the body.

The addition of specific growth factors to the culture medium, 3D culture and the use of bioreactors are combined to achieve a tissue matured in vitro that will readily integrate into the host tissue once transplanted into the recipient.<sup>18</sup>

## Angiogenesis

After the transplant, the new tissue needs to integrate in to the host. In this process it is essential that connections are established with the recipient's vascular system. These connections should be made by invasion of endothelial vessels from the tissue surrounding the implant. The period of time between the transplant and the endothelization of the new tissue, during which the tissue must be fed through diffusion, varies. This fact limits the thickness of the tissues produced through TE to few millimetres.<sup>19</sup> In thicker tissues, nutrients and oxygen will not reach the central part sufficiently quickly and this will be lost by necrosis. The lack of a vascular bed is the greatest limitation of TE in the production of organs and tissues. In order to improve the vascularisation of the TE tissues, three classical strategies are followed:

1. In vitro contribution to the endothelial cells prototype. When endothelial cells are cultivated in 3D, they spontaneously form tubular structures similar to tissue endothelium. Such cells can be cultured, expanded in vitro and seeded on the prototype in order to generate a vascular matrix that will accelerate the endothelization of the tissue once it has been transplanted. Endothelial cells can be obtained from bone marrow precursors or small veins. Given that these cultures are slow and inefficient, the effectiveness of this endothelization system is more theoretical than practical. Note that it is not possible to use cells from the donor given that endothelial cells are very immunogenic.
2. To enrich the pre-implant prototype with pro-angiogenic factors. Factors such as VEGF or PDGF can be added, producing a fast endothelial response from the tissue bordering the site where the TE prototype is transplanted. These peptides do not have a very long lasting effect due to their relatively short mean lifetime. However, growth factors can be produced by the cells of the synthesized tissue, either physiologically or accelerating its production through gene therapy.
3. It is possible to enrich the bed where the tissue is grafted through the creation of small arteriovenous fistulas that will place the transplanted tissue closer to the vessels, facilitating faster vascularisation. This has been carried out successfully in experimental transplants.

These strategies could be used together to accelerate the endothelization of the tissues produced by TE. Although such a combination might produce fast endothelization, there will always be a nonvascular stage that will restrict the original thickness of the implant, and currently this limits the potential of developing complex organs in the laboratory.

Recently, two new approaches have been described in the literature that may solve the problem of vascularisation of tissues produced using TE techniques:

1. Organ printing technology, described above, allows vascular trees to be designed. These structures can be introduced into the tissues and organs during scaffold assembly to facilitate the subsequent vascularisation of these tissues. This type of tool represents a great advance in the area of Tissue Engineering, although, unfortunately they are not yet available to clinical laboratories.
2. The use of the native vascular trees obtained by decellularization.<sup>20</sup> In theory, using this system, the decellularized organs would maintain the vascular tree, from the entry artery to the exit vein and all the capillary system. In the future, the use of this strategy may solve the problem of vascularization of complex

bioartificial tissues and organs, although at the moment only small prototypes can be developed with this technology.

## **TISSUES SYNTHESIZED USING TISSUE ENGINEERING TECHNIQUES**

Given the current limitations of TE, in the laboratory it is only possible to produce tissues which are simple both in structure and function. The *in vitro* synthesis of complex organs is still very far from a reality. In the following pages, we will describe the most clinically useful tissues types in this area.

### **CARDIOVASCULAR SYSTEM**

Cardiovascular diseases of the heart and peripheral blood vessels as well as those affecting valvular system, are the most common illness in the developed world. Current treatments can address most of these problems, but not all of them. In these cases, TE can offer therapeutic alternatives.

Myocardial ischaemia is the most common cause of death worldwide. Ischaemia associated with coronary artery obstruction results in the death of contractile cells in the area, while chronic lesions due to arterial hypertension produce a progressive reduction in the number of cardiomyocytes. Although the heart is a muscle, it does not have the same capacity for regeneration as striated muscle. For this reason, the acute or chronic loss of a critical number of cardiomyocytes leads to heart failure. The only solution when this becomes a serious problem would be a heart transplant.

The technology necessary to create an organ as complex as the heart does not yet exist. However, a bioartificial heart capable of contracting has been produced from the decellularized matrix of a whole heart subsequently seeded with cardiomyocytes. In the medium to long-term, this may be an alternative for heart transplantation.<sup>16</sup>

However, currently, what can be attempted is to regenerate damaged heart through cell therapy. This technique involves the implantation of cells in the heart that will improve the decreased contractile function.<sup>21</sup> In this therapy, both experimentally and clinically, cells from various different sources have been used: Myoblasts cultured from skeletal muscle, progenitor cells extracted from bone marrow, embryonic stem cells, and myocardial stem cells. These cells can be re-introduced into the cardiac muscle, in various ways: Through a direct injection into the injured area or via the blood stream. The use of this cardiac cell therapy is on increase. However, while it seems to be a safe therapy, there are still doubts about its effectiveness.

Although at the moment TE does not offer many solutions for the muscle, the technology for valves is more advanced. Despite their complexity, valves are structures which are relatively easily constructed outside the body. There have been synthetic mechanical valves for long time, offering considerable benefits and excellent clinical outcomes. However, they do have two disadvantages: The patient needs to take anticoagulants and they are susceptible to infections. Mechanical valves can be replaced by biological valves, coming from animal tissue (bovine or porcine), or homograft valves from cadavers. Both options avoid the need to take anticoagulants and are less susceptible to infections. However, these biological valves have a limited life and must be replaced after a period of time. In particular, the use of biological valves is more problematic in children and

teenagers, since besides continuing to function, they must be able to adapt to the growth of the patient. A biological valve with living cells from the patient, responsible for tissue repair following damage caused by mechanical stress, should have an unlimited life and adapt to the growth process without difficulty.<sup>22</sup>

There are two different strategies for TE of heart valves. Both are based on the design of a scaffold similar to the valve to be replaced. In the first case, an acellular scaffold is transplanted and subsequently populated with precursor cells (endothelial and mesenchymal) that re-organize the provisional structure and synthesize the natural matrix of the valve. This model has been used in experimental transplants and in clinical practice with mediocre results.<sup>23</sup>

The second strategy is *in vitro* seeding of the scaffold. This would take place in a bioreactor that imitates the physiological and mechanical conditions to which valves are subjected. After this phase, the valve would be implanted and undergo a new process of re-organisation *in vivo*. A model based on the PGA in combination with poly-4-hydroxybutyrate and seeded with endothelial autologous cells has been trialled experimentally. Twenty weeks following the transplant, the valve continued to function. The original scaffold was partially degraded and there were glycosaminoglycans and collagen fibres inside the valve.

So far, it is difficult to justify the use of valves produced by TE in humans because a malfunction would be life threatening. However, in those cases in which there are no alternatives (e.g., exceptional paediatric cases) the use of these types of valves might be considered.

Another important aspect of cardiovascular TE is the production of blood vessels. Studies of the use of synthetic materials to make arterial channels for revascularization surgery have been going on for fifty years. Nevertheless, the best outcomes are currently achieved using autologous vessels (e.g., the saphenous vein or internal mammary artery). Synthetic vessels, such as those constructed using ePTFE or Dracon, have been used successfully for decades. However, synthetic vessels made of these materials present various types of problems: Thrombosis caused by the lack of a functional endothelium, restenosis due to chronic inflammation and/or poor compliance, as well as susceptibility to infections.<sup>24</sup> These problems become more pronounced when the vessels to be replaced are narrow (4-6 mm diameter) given that they have a poor permeability in the longer term. In these cases, when there is no suitable autologous material, the production of vessels through TE techniques that improve on the current artificial vessels has a wide field of application.

In the clinical field there are three different ways to approach the production of blood vessels:

1. Endothelization of conventional prostheses. Endothelium is cultured using cells harvested from a small biopsy of a vein. This is seeded on an ePTFE or Dacron prosthesis which, after a period in culture, is transplanted. In this way, a conventional vascular prosthesis with endothelial function is obtained. Some studies have shown that this type of prosthesis could have a long-term permeability similar to that obtained using the autologous saphenous vein grafts.<sup>25</sup>
2. The production of vascular segments by seeding cells on a biodegradable scaffold. A PLA/PCL tubular scaffold reinforced with a PGA ring has been used. This scaffold, seeded with autologous cells from the bone marrow, has been trialled in the outflow tract of the pulmonary artery in paediatric lesions,

with excellent results.<sup>26</sup> Follow-up of the patients has revealed that these vessels have increased in size as the children grow, resolving one of the problems presented by inert materials.

3. Development of vascular segments by the in vitro production of the scaffold with the patient's own cells.<sup>27</sup> The prototype consists of a vessel made of three different layers: An adventitial layer, an internal acellular membrane and an internal endothelial layer. The internal membrane is produced using 2D culture of sheets of dermal fibroblasts. These cells produce a collagen-rich extracellular matrix. Subsequently, these fibroblast sheets are wrapped around a metallic axel and cultured for 10 weeks, stimulating their fusion. This scaffold is dehydrated and forms a tubular acellular substrate. New layers of living fibroblasts are added on the external surface to form the adventitial layer. Finally, endothelial cells are seeded on the internal surfaces. With this approach, it has been possible to build completely autologous arterial segments. These have been successfully tested in arteries, in particular, for the creation of arteriovenous fistulas. Building on this technique, scaffold formation by cells themselves, other researchers have succeed in producing vessels with various layers of a normal vessel (muscular and tunica adventitia).<sup>28</sup> The experimental transplantation of these prototypes has given promising results.

These three clinical examples show us that in the quite near future, it will be possible to provide living arterial vessels for clinical use with better properties than those used to date.

## LUNGS AND RESPIRATORY TRACT

Lung diseases cause around 400.000 deaths per year in the United States. Lung regeneration is limited and if the damage is extensive, the only solution is lung transplantation. The lung is a very complex organ with close contact with the airways and blood vessels for the gas exchange. In order to rebuild a lung, in addition to a complete vascular tree, an airway tree is required, from the large airways (trachea, bronchi) to the bronchioles and alveoli. A prototype artificial lung has recently been achieved using decellularized lung matrix and vascular tree, seeded with cultured lung epithelium and endothelial cells respectively. This organ, maintained in a bioreactor, was found to contain lung epithelium more or less like to normal epithelium and showed good endothelization. The mechanical characteristics of the new lungs were very similar to those of natural lungs. The lung, once transplanted to the experimental animal, participated in the exchange of oxygen.<sup>29</sup> Although the design is still far from ready for use in clinical practice, it represents a qualitative step forward with great potential for future development.

Another very interesting area is the regeneration of the airways. The growth of tumours and chronic infections may require resection of large segments of trachea and bronchi. Prostheses have been made with inert material but these tend not to be able to provide the resistance and elasticity of the natural airways, and their lack of epithelium may be behind the appearance of inflammatory granulomas, which hinder the normal circulation of air. A living tissue graft, retaining the natural cartilage of the airways and the epithelium that removes particles deposited in the bronchi, may be a solution for

some of these patients. An approach to airway regeneration which has already been tested clinically is the use of a noncellular tracheal matrix seeded with chondrocytes derived from mesenchymal stem cells and airway epithelium. The cells, once seeded into the matrix, mature in a bioreactor. A trachea regenerated in this way has been successfully used in clinical practice.<sup>30</sup>

## HEPATIC TISSUE

The now standard treatment for terminal liver failure is liver transplant. Current outcomes following liver transplants are excellent, however, the low number of organs available, the high cost of the transplant and the requirement for lifelong immunosuppressive therapy, are major drawbacks of this technique.<sup>31</sup> The limitations are even clearer in the case of acute liver failure, in which, if the patient survives the liver insufficiency, the liver should be able to completely regenerate. In these cases, the liver transplant, which is indicated due to the high mortality associated with acute liver failure, could be avoided if a short-term solution were to be available, such as temporary liver replacement.

The liver is an organ with important synthetic and metabolic functions. Specifically, it carries out more than 500 functions that are difficult to replace using artificial methods. However, in some diseases the liver has only a partial deficit in certain of these functions. The treatment of these partial diseases using liver transplant could also be avoided with the development of artificial livers. These considerations have boosted research in the area of liver support systems, which would be able to replace the function of the damaged organ.

Given that the liver is a very complex structure to be produced in the laboratory, hepatic TE focuses on the development of extracorporeal liver supports and treatments for partial deficiencies of this organ. However, despite substantial research effort, basic aspects of liver therapy are far from being solved.

Completely artificial (acellular) liver support systems are on the market. These systems have the ability to eliminate toxic substances, improving the clinical and biochemical parameters of the patients. However, they lack biosynthetic activity and have not yet shown improvements in the survival of the patients suffering from acute liver failure.<sup>32</sup>

Bioartificial liver (BAL) support systems are devices that rely on the functionality on living cells.<sup>33</sup> They normally use porcine hepatocytes, immortalized human cells and/or cells derived from hepatic tumour cell lines; the choice of these different types of cells will be discussed below. Since hepatocytes are cells that require being adhered to survive; they must be associated with some type of support. For this, hepatocytes can be entrapped in gel, immobilized inside collagen-coated culture dishes, and cultured on and in semi-permeable fibres as well as in three dimensional porous matrices. These systems operate as extracorporeal bioreactors that replace the liver function. There are various examples of bioartificial liver support systems: HepaAsist (Cedars-Sinai Medical Center, Los Angeles, California, USA), AMC (Amsterdam Medical Centre)-BAL, MELS (Modular Extracorporeal Liver Support, Charite, Berlin, Germany), ELAD (Extracorporeal Liver Assist Device, Vital Therapies Inc. San Diego, California, USA), and BLSS (Bioartificial Liver Support System, Excorp medical Inc. Minneapolis, Minnesota, USA). However, few of these devices have gone beyond Phase I clinical trials. The results obtained with them are variable; generally they improve the patient's clinical and metabolic condition, but again they do not improve survival rates.<sup>32</sup>



## Hepatic Tissue Engineering

The hepatocyte transplants could also be used in the treatment of some liver lesions, especially those due to single enzyme deficiency. This approach allows us to work with cells instead of whole organs, which offers several advantages:<sup>31</sup>

1. Cells can be expanded *in vitro* until they reach a critical volume to be transplanted. The transplantation of a hepatocyte mass equivalent to 10% of the patient's liver can be enough to normalize its metabolic situation in cases of enzyme deficiency.
2. These cells expanded *in vitro* can be genetically or immunologically manipulated before being transplanted. In cases of allogeneic transplants, donor liver cells can be immunomodulated in order to avoid an immune response in the recipient.
3. Hepatocytes isolated from the whole organ can be easily cryopreserved in a biobank, until required by a patient.
4. Several patients could be treated with a single donated organ.

There are currently several sources of cells with potential for use in liver tissue engineering including: Porcine hepatocytes, human adult hepatocytes, hematopoietic stem cells, progenitor (oval) cells, and cell lines derived from hepatoblastoma. In addition, it has recently been observed that mesenchymal stromal cells (MSC) are able to differentiate into hepatic precursor cells *in vitro*. However, the efficiency of the culture is very low, except in the case of tumour cells. Indeed, the advantage of using cell lines derived from tumours is that they can be readily cultured to produce a large number of cells, but this source would present a safety problem in clinical applications. The use of porcine hepatocytes is also controversial, given that they could provoke an immune response in the patient or transmit some type of retrovirus. On the other hand, the use of normal human hepatocytes is limited due to difficulties in propagating them *in vitro* and also because when cultured in excess, these cells undergo a differentiation process, losing the phenotype of hepatic cells.

Donor hepatocytes have been directly injected into the portal vein in order to treat patients suffering from Type I Crigler-Najjar syndrome.<sup>31</sup> However, when a large number of cells are transplanted, some complications arise, including portal hypertension, portal vein thrombosis and pulmonary embolism. An alternative to hepatocyte infusion could be to implant hepatic cells fixed to a scaffold that allows them to carry out their physiological function and to differentiate. This strategy would allow the transplantation of a thin tissue, with the ability to integrate in the tissue of the recipient and replace the metabolic damaged functions. This model does not aim to regenerate a whole organ, but presents a therapeutic alternative to address certain pathologies, avoiding the transplantation of an entire organ.

Finally, attempts have been made to construct a whole liver using TE techniques based on decellularized liver tissue seeded with liver cells. So far, using this procedure, which allows the entire liver vascular and biliary tree to be preserved, it has been demonstrated that the engineered liver at least momentarily is able to take on some of the functions of the mature organ after experimental transplantation (production of albumin and conjugated bilirubin, among others). This step represents an important milestone in progress towards the future creation of complex organs.<sup>34</sup>

In short, it is necessary to find therapeutic alternatives to the solid organ transplantation. In some cases, bioartificial liver (BAL) support systems and the transplant of hepatocytes produce very good outcomes, but they still have severe shortcomings. The definitive solution



requires identifying an optimal source of cells and managing to propagate hepatocytes in culture in large enough quantities to obtain a critical mass suitable for use in the various strategies mentioned. In relation to this, Tissue Engineering offers great possibilities, although the technology must be improved before functional liver tissue can be obtained.

## RENAL AND GENITOURINARY TISSUE ENGINEERING

### Kidney

The kidney is a complex organ that carries out functions vital for the body. Renal failure is a condition that in the terminal stages needs to be treated with dialysis (peritoneal or haemodialysis) or transplantation. Current dialysis techniques are very efficient but not very adaptable; and tend to be associated with high morbidity for the patients.<sup>35</sup> Accordingly, a kidney transplant is currently the best therapeutic option for patients suffering from terminal renal failure. However, the limited availability of compatible donor organs and requirement for lifelong immunosuppressive therapy still represent two critical obstacles. Further, given the anatomical and functional complexity of the kidneys, currently we are not able to create a complete functional organ in the laboratory.

The renal TE of the kidney is currently less advanced than in other tissues due to the existence of effective clinical alternatives. Nonetheless, there have been trials using haemodialysis machines combined with cultured cells from the proximal tubule (RAD: Renal Assist Device), which improve on the characteristics of classical haemodialysis machines.<sup>36</sup> On the other hand, there are experimental models showing improvement in the function of a damaged kidney based on cell therapy. These models use cells from various sources including ESC, MSC, and AFS which injected into a damaged kidney can improve its functionality.<sup>6</sup> However, these alternatives are still far from being used in clinical practice.

### Genitourinary Organs

By comparison, the production of genitourinary tissue is more developed. Genitourinary tissue reconstruction following major oncological surgery or congenital lesions is of great therapeutic importance. For the reconstruction of a defect or lesion of the genitourinary tissue, it is possible to use:

1. Autologous non-urinary tissue (skin, gastrointestinal segments, mucosa).
2. Urologic allogeneic tissues from a cadaver donor.
3. Heterologous tissues or substances (bovine collagen)
4. Artificial materials (silicone, Teflon, polyurethane)

However, given that all these alternatives present problems, none of them provides a definitive solution. The goal of genitourinary tissue engineering is to achieve functional equivalents to replace the damaged or lost urinary tissue.

The scaffolds most commonly used in genitourinary TE are: Natural materials (collagen, alginate), acellular natural matrices (bladder submucosa, small intestine submucosa—SIS) and synthetic polymers (PGA, PLG). The advantage of synthetic polymers is that they can be designed in a controlled way in order to achieve the desired properties. However, natural materials tend to integrate better into the recipient.

## **Urethra**

Several strategies to regenerate urethral tissue have been proposed during recent years. The transplant of cultured urethral epithelium has been used in clinical practice for the treatment of hypospadias.<sup>37</sup> Other models based on extracellular matrices are also being used. For example, there have been experimental transplants of intestinal submucosa (SIS) and PGA scaffolds, as well as urethral and bladder acellular matrices. Urological acellular matrices offer significant advantages over autologous tissue for the repair of lesions, given that it enables the surgical process of collecting the tissue to be used as the graft to be avoided, reducing the morbidity of the donor site. Following this strategy, acellular matrix based on collagen from the human bladder has been successfully used to repair urethral defects.<sup>35</sup>

## **Bladder**

The most common approach for repairing damaged or lost bladder tissue involves using gastrointestinal segments. However, this tissue is designed to absorb solutes. Due to this physiological difference, various complications may occur when it is used in the bladder, including infection, metabolic complications and perforation. Therefore, during recent years, therapeutic alternatives for the repair in this organ have been investigated, such as the use of physical techniques of bladder expansion and reconstruction in experimental models with seromuscular grafts combined with fragments of de-epithelialized large intestine.

Allogeneic or xenogenic acellular matrices have been also used. These have been used to serve as a guide to help bladder cells invade the matrix and completely regenerate the bladder walls. However, the outcomes of the experimental transplant of such matrices were poor. Although the bladder epithelium was able to invade and to cover the entire acellular matrix, rebuilding of the muscular layer, essential for its correct function, was not achieved.

Better results have been obtained with matrices seeded *in vitro*. In dogs an artificial bladder has been created using tissue engineering techniques. It was based on a biodegradable polymeric matrix seeded with autologous urothelial and smooth muscle cells. During eleven months of monitoring, this neo-bladder was able to maintain its flexibility, hold urine and also had normal histological architecture. A very similar model based on a collagen/PGA scaffold seeded with urothelial and muscle cells previously expanded using cell culture, is being used in human therapy with acceptable results.<sup>38</sup>

## **Incontinence and Vesicular Reflux**

Both incontinence and vesicoureteral reflux are very common conditions of the genitourinary system. Both are due to local muscle weakness. The most common treatment for these pathologies is the injection of a substance that provides the zone with more resistance (collagen, Teflon, etc.). The ideal substance for this technique would have the following characteristics: It can be injected, does not migrate or produce an antigenic reaction and is safe for use as a therapy.<sup>2</sup> In this context, autologous chondrocytes, cultured and embedded in alginate gel, have been successfully used in the treatment of these conditions.

## Genital Tissues

Sometimes, due to congenital malformations or other clinical conditions, penis reconstruction is needed in order that the patient can recover normal morphology and functionality. Several different approaches have been used to achieve this reconstruction. The options offered by tissue engineering are the most appealing, given that they use autologous rather than synthetic material, reducing the risk of rejection.

In rabbits, there is a reconstruction model for the cavernous body of the penis, using acellular matrix based on collagen of the cavernous body. Endothelial and smooth muscle cells are seeded on this and the ensemble is transplanted. Animals recovered their erectile function, and with it their ability to mate and breed, one month after the transplant.<sup>35</sup>

The most common prostheses are silicone-based; this, however, involves a degree of risk given that it is not a very biocompatible material. By comparison, a prosthesis made of autologous cells would be a much better solution. There have been attempts to create penile prostheses in rabbits, based on autologous chondrocytes seeded on cylindrical PLGA matrices, providing good functional results two months after the transplant.

With respect to female genital organs, there are several different lines of research. On the one hand, there have been attempts to synthesize a uterus using tissue engineering in rabbits, seeding epithelial and smooth muscle cells on a biodegradable polymer matrix. Six months after the transplant, histological analysis showed the tissue to be normal and the function of this artificial uterus is currently being studied.

On the other hand, a similar strategy has been followed in order to try to construct an artificial vagina through tissue engineering. To achieve this, vaginal epithelial cells and smooth muscle cells were seeded on a biodegradable scaffold and the ensemble was transplanted into mice. Immunohistochemical studies showed that the phenotype of the regenerated tissue was vaginal.

## TISSUE ENGINEERING IN THE AREA OF TRAUMATOLOGY

Orthopaedics is one of the most interesting fields for tissue engineering. Basically, this field looks to address three requirements: Bone repair, cartilage repair and generation of tendons.

### Bone

Bones have a great healing capacity and most lesions recover by osteosynthesis and immobilisation. However, some lesions need measures to complement the spontaneous regeneration of the bone. In these cases, the repair of bone defects can be carried out using bone grafts. Autologous cancellous bone grafts are the treatment of reference to improve a bone defect and it is the material with the highest osteogenic capacity. However, the clinical use of autologous bone is limited due to the considerable morbidity at the donor site. Bone allografts and xenografts are normally used when autologous tissue cannot be employed. Other biomaterials include acrylic cements, calcium phosphate and ceramics based on hydroxyapatite (animal or synthetic origin). In addition, osteoinductive agents, such as platelet-rich plasma and BMPs (bone morphogenetic proteins), can be used to

strengthen the repair of bone lesions. Despite this therapeutic arsenal, some bone lesions do not fully recover.

Tissue engineering offers therapeutic alternatives in these cases.<sup>39</sup> Specifically, osteoprogenitor cells are used in combination with various types of scaffolds.

### **Bone Tissue Engineering**

In the case of bone tissue, cells from a wide range of sources can be used safely. Large quantities of osteoblasts can be cultured from tissue harvested from spongy bone biopsies. It is also possible to culture from periosteal cells, bone marrow and other mesenchymal tissues such as adipose tissue,<sup>8,13</sup> which have bone regenerative capacity. There is also a wide range of scaffolds that can be used in bone TE techniques. Two models based on the use of hydroxyapatite have been used in clinical practice:<sup>39</sup> One uses cells expanded from bone marrow for the treatment of fractures of the tibia, humerus and ulna; while the second seeds autologous periosteal cells on this material for reconstruction of the thumb. Despite these promising results, so few patients have been treated so far that it is difficult to assess the effectiveness of these alternative approaches. More progress needs to be made in this field (more extended series of treated patients, optimization of culture and implant techniques, improvements in the available biomaterials, etc.) before these techniques can displace the conventional treatments available to date.

### **Cartilage**

Cartilage is a tissue that, in contrast to bone tissue, lacks regenerative capacity. Severe cartilage lesions do not regenerate. Currently, there are no surgical techniques which enable “*restitutio ad integrum*” of a cartilage injury.

Since 1994, when Brittberg et al<sup>5</sup> described an autologous chondrocyte transplant, cartilage tissue engineering has made great progress. Normally, autologous articular chondrocytes from a nonweight-bearing region are used as the source of cells. To achieve this, a biopsy is required, provoking morbidity in the donor area. In order to avoid this, other sources of autologous chondrocytes are being studied, in particular, nasal and auricular cartilage, from which biopsies can be taken in a less invasive way. Another accessible source of cells for articular cartilage repair is mesenchymal stem cells,<sup>17</sup> from the bone marrow or adipose tissue, which are able to differentiate into chondrocytes given suitable conditions.

The technique described by Brittberg involves transplants of a chondrocyte suspension culture directly onto the lesion. This transplant system presents surgical problems (open surgery, loss in volume transplanted, need for a periosteal patch). These problems could be avoided with the associated use of a scaffold. Several materials have been described and used in experimental transplants, including biodegradable polymers and hydrogels. Since 2000, Type I and II collagen scaffolds have been used in clinical practice, with chondrocytes seeded at the end of the expansion period. The use of this new technique makes surgery easier and the outcomes are similar to those obtained with the traditional transplant, but so far there have been no serious comparative studies.

To summarize, chondrocyte transplantation is accepted as a consolidated therapy. Studies comparing this approach with other techniques (e.g., microfractures, mosaicplasty) indicate that it has clinical advantages. However, the cost-effectiveness of this therapy and the long-term evolution of treated patients are still unknown.<sup>40</sup>

A solution to its current limitations may come from a combination of the cells, scaffolds and bioreactors. In particular, the application of pressure to the cells produces *in vitro* synthesis of an extracellular matrix that is closer to the normal matrix. This would make the implant easier (it could be carried out through simple arthroscopy) and would probably improve the results.

## **Tendon**

Articular tendons have a poor capacity for self-repair. Current options to address a lesion in this tissue are: (a) transplant of autologous tissue, with the associated morbidity of the donor area; (b) transplant of allogeneic tissue, with the risk of infections transmission and immune rejection; and (c) implantation of synthetic materials, with poor medium- to long-term results.

These issues, with the consequent need to find a tissue that would overcome for the limitations of the existing therapies, have encouraged research in tissue engineering of tendons to make great advances.

Fibroblasts or mesenchymal stem cells can be used as a source of cells for TE of tendinous tissue. On the one hand, fibroblasts can be obtained from any mesenchymal tissue and they are able to proliferate, synthesize and organize the collagen that they produce. On the other hand, adult fibroblasts preserve most of the phenotypic characteristics necessary to carry out repair of the tendons. However, these are relatively quiescent cells that have limited potential for subsequent differentiation into specific fibroblasts of tendinous tissue.<sup>41</sup>

The other cell source is the MSCs present in the bone marrow. These cells are easy to obtain, have great proliferative ability, are able to differentiate into any cell type with mesenchymal origin and adapt well to the conditions of the growth niche.

Collagen has been one of the materials most studied for TE of tendinous tissue, given that it is one of the main components of the natural extracellular matrix of tendons. Bovine collagen has been used, alone or seeded with human cells. Although collagen scaffolds are able to favour adhesion and cellular proliferation, they do not have great mechanical strength. Besides, it presents rejection problems given that it produces certain immune reaction in the recipient.

A material with better mechanical characteristics that has been tried is silk (indeed, it was first used in clinical practice a long time ago as a suture). As well as offering excellent mechanical properties and a known degradation behaviour, silk is a cheap and biocompatible material. *In vitro* trials it has been shown that it allows adhesion, growth and cell differentiation.

Another option is polymers (PGA, PLA, PLGA, etc.). These polymers are gradually re-absorbed and in this way the immune response that they could produce is minimised. In addition, they can be designed with made-to-measure characteristics: Degradation rate, strength, shape, size, etc. Most of these polymers are already in common use in clinical practice and *in vitro* it has been shown that they allow fibroblast adhesion and proliferation on their surface.

Unfortunately, none of the scaffolds employed to date are able to support the mechanical forces born by a natural tendon. The organization of the collagen synthesized by seeded cells is far from being ideal for these mechanical requirements and producing a working tendon using TE is still a distant possibility.

## SKIN

The skin is the organ that isolates us from the external environment. This function is essential for life and the loss of large surface areas of skin (e.g., on burned patients) carries a high mortality. If the injury is small, the wound can heal from the borders or from underneath. However, when it is extensive and/or deep it has to be sealed using a skin transplant and the only skin that can be used is that of the patient themselves. A donor skin transplant would be rejected and the use of immunosuppressors to avoid the rejection is contraindicated given that such patients tend to suffer serious infections. Normally, thin laminae of meshed autologous skin are transplanted in order to increase the area covered. Despite this technique, when the burn affects more than 75% of the total body surface area, there is not enough donor area to obtain autografts. In these cases it is necessary to turn to TE techniques to generate sufficient coverage.

An effective method for epithelium culture from a skin biopsy was not developed until 1975.<sup>42</sup> Since then, this culture system has been used in the treatment of large burns.<sup>3</sup> The culture method, developed by Rheinwald and Green, produces laminae of epithelium for transplant onto the wound and this technique helps to increase between 3.000 and 5.000 times the initial surface of the biopsy. Following the transplant, the epithelium lamina must graft on the bed of the wound, develop an effective basal membrane, reproduce the entire epithelium differentiation program and continue the epithelisation for the rest of the patient's life. The weaknesses of this technique are the extreme fragility of the cultured epithelium, as well as poor engraftment of the transplanted cells and the dermo-epidermal interface.

In order to improve these results, cultures of keratinocytes have been combined with the transplant of skin components (artificial or skin from a cadaver donor). More recently, systems of complete cultured skin (dermis and epidermis) have been developed, improving on the outcomes with isolated epithelium.

The epithelial component of the skin is mainly made of cells, whereas in the dermis the extracellular matrix dominates. This skin dermal matrix is mainly composed of collagen, elastin and fibronectin, among others and the various different strategies of skin TE are mostly focused on production of this dermal component. Collagen and fibrin are the most commonly used materials as a source of extracellular matrix for skin TE, while dermal fibroblasts are used as a cell source. The matrix-fibroblasts combination generates a dermal analogue that stimulates the growth of the keratinocytes on the surface, their stratification and the *in vitro* generation of a functional dermo-epidermal interface.<sup>43</sup>

These models of skin produced through TE are now routinely employed in the treatment of large burns, chronic skin ulcers, giant nevi, etc.

Although the results obtained can be considered satisfactory, given that they achieve their main objective of providing the body with protection from the external environment, the aesthetic and functional outcomes are mediocre and need to be improved.

Tissue Engineered skin may also be used both for the study and palliative treatment of some genodermatoses. When skin is created from biopsies of patients suffering from a type of genodermatosis using these techniques, it is possible to reproduce the disease by transplanting the skin graft onto an athymic mouse. In this way it is possible to create an animal model of a rare human disease.

The current techniques allow us to obtain and culture these pathological cells. On the one hand, such cells may be modified by gene therapy to correct the defect caused by the disease. These corrected cells could then be used in bioengineered skin models for treating the lesions of this type of patient. On the other hand, the pathological cells can be used alone or together with healthy allogeneic cells (chimeric skin) for the palliative treatment of these patients.

These culture techniques and the development of artificial skin can be applied to other types of epithelia (oral and vesical mucosa, among others) and in the regeneration of the epithelium of the eye.<sup>44</sup>

One of the limitations of TE can be clearly observed in this tissue. Despite more than thirty years of development, it is only possible for us to recover the lost function, but we are not able to synthesize skin completely like the natural-type, given that we do not have the technology to produce the cutaneous appendages (glands, hair follicles, etc.). Even with these limitations and the need for improvement, skin tissue produced using TE is regularly used in clinical practice and does improve the morbidity and mortality associated with large burns.

## NEURAL TISSUE ENGINEERING

Because lesions in nervous tissue being very common, repair of this type of tissue is one of the most important objectives of TE. Traditional therapeutic alternatives offer limited solutions and the consequences of the lost function are dramatic. The pathophysiology of the repair process is completely different in the central and the peripheral nervous system. In particular, while in the peripheral nervous system there is some degree of repair response, in the central nervous system repair is largely inhibited.

A lesion in a peripheral nerve involves the interruption of the connection between the central nervous system and the zone innervated by the nerve. This produces motor and sensory changes that depend on which nerve is affected and the extent of the lesion. If there is minimal damage (neuropraxia), the repair is carried out spontaneously and quickly. However, if the lesion is more extensive and there is axonal damage, the axon distal to the lesion degenerates and the repair is carried out through the growth of an axon from the area proximal to the lesion towards the end of the nerve, producing a deferred repair. In this process, it is essential that the nervous sheath remains intact and the Schwann cells are involved. These glial cells guide regeneration and provide neurotrophic factors that promote the growth of axons. When the nerve has been completely damaged, surgical repair of the nervous sheath is required in order that the repair process can occur. When the lesion is extensive and there is loss of tissue, the autologous transplant of a nerve is needed in order to complete the communication between the sectioned parts. This type of transplant is limited due to a shortage of the tissue, given that we have very few nerves that can be removed from a zone without causing an substantial lesion. Sensory nerves, such as the sural nerve, are used to carry out this kind of repair.

TE could provide an alternative to the use of autologous nerves as it is possible to synthesize scaffolds that operate as a bridge in a damaged nerve. This strategy has been used with limited success, given that without trophic factors, the growth of the axons is minimal. It is necessary to provide a suitable environment to make the axons proximal



to the damaged nerve start their growth and repair the lesion. In relation to this, various different cell types involved with these channels have been used (including Schwann cells, genetically modified fibroblasts and mesenchymal stem cells). In experimental animals, better outcomes have been obtained, although they too were very limited. Indeed, the clinical use of these types of technologies seems to be very far off.<sup>45</sup>

In the case of central nervous system repair, the problem is even more complicated. The repair process of the lesions produces a scar that physically blocks growth of axons. Moreover, in the CNS there is not only the need for a bridge that regenerates the nervous conduction, but also the neural body can be damaged by the process. Using TE techniques, we can try to replace the damaged or lost tissue like in any other tissue, that is, using cells and scaffolds. Another possibility offered by tissue engineering is the use of scaffolds that contain neurotransmitters; these are control released, in order to treat neurodegenerative diseases such as Parkinson's.<sup>46</sup>

The limitations of TE in this tissue are more severe than in any other tissue, given that any minimal inflammation produced by the process of implantation/re-absorption would cause more harm than good.

Therapeutic approaches based on cell therapy have been proposed for the treatment of vascular lesions in the brain.<sup>47</sup> In preclinical studies, endothelial progenitor cells and embryonic stem cells have been used. There is also some clinical data on intravenous infusion of mesenchymal cells and cultured neural stem cells. The results obtained to date demonstrate at least that such types of treatment seem to be safe.

One of the most critical challenges for regenerative medicine is how to address spinal cord injuries. It is estimated that there are more than 2.5 million people worldwide suffering from a spinal cord injury. However, at the moment there is no therapy able to regenerate seriously damaged spinal cord.<sup>48</sup> When the spinal cord is injured by trauma, there is a response that tends to result in cell death of both the neurons and the glial cells. The scar produced is often perpendicular to the medullar axis and, moreover, it contains substances that are axon-growth inhibitors. Several trials have been made using cell therapy to set up an axon bridge able to stretch across this scar tissue.

Cells and tissues from a variety of sources have been used both clinical and experimentally for the treatment of spinal cord injuries including peripheral nerves, Schwann cells, olfactory ensheathing glia, embryonic nervous tissue, hematopoietic stem cells and activated macrophages. Although some of the results in experimental animals are promising, few trials have been carried out in humans; there are neither groups of homogeneous patients nor comparative studies and, what is more, none of the trials have produced spectacular results.

## CONCLUSION AND FUTURE CONSIDERATIONS

As previously commented in this chapter, tissue engineering is a very recent discipline which sets out to offer solutions to clinical problems that cannot be addressed using any other methodology, but, as present, is of limited success. The current boom TE is experiencing both scientifically and in terms of media coverage is due to expectations about its future. Tissue Engineering will surely become a part of routine therapy in coming years and will help to resolve problems that have no solution using conventional therapies.

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